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PREFACE

With pleasure and in sincere appreciation of their cooperation we thank our many colleagues who served as authors of this volume. We trust that in so doing we may speak also for their fellow biochemists the world over. If the good work of any has failed to receive mention in the pages that follow, perhaps we can attribute such omissions to the rather embarrassing and frustrating restrictions with which we encompass our colleagues—tight assignments of space and close time schedules. With a desire to encourage reviews that are critical and analytical we also advise an author to select only a portion of the papers that might properly fall within the subject of his review—concentrating upon those which indicate the major trends of research and which are of greatest help in evaluating the present status of the subject.

We express our thanks also to E. L. Smith who has served as a member of the Committee for the past five years. He is succeeded by C. B. Anfinsen. The editor has been granted a two-year leave of absence and during this time, with the continuing assistance of F. W. Allen and G. Mackinney, E. E. Snell will serve as Editor. W. C. Cutting, Editor of the Annual Review of Pharmacology, has accepted the interim appointment of Managing Editor of Annual Reviews, Inc.

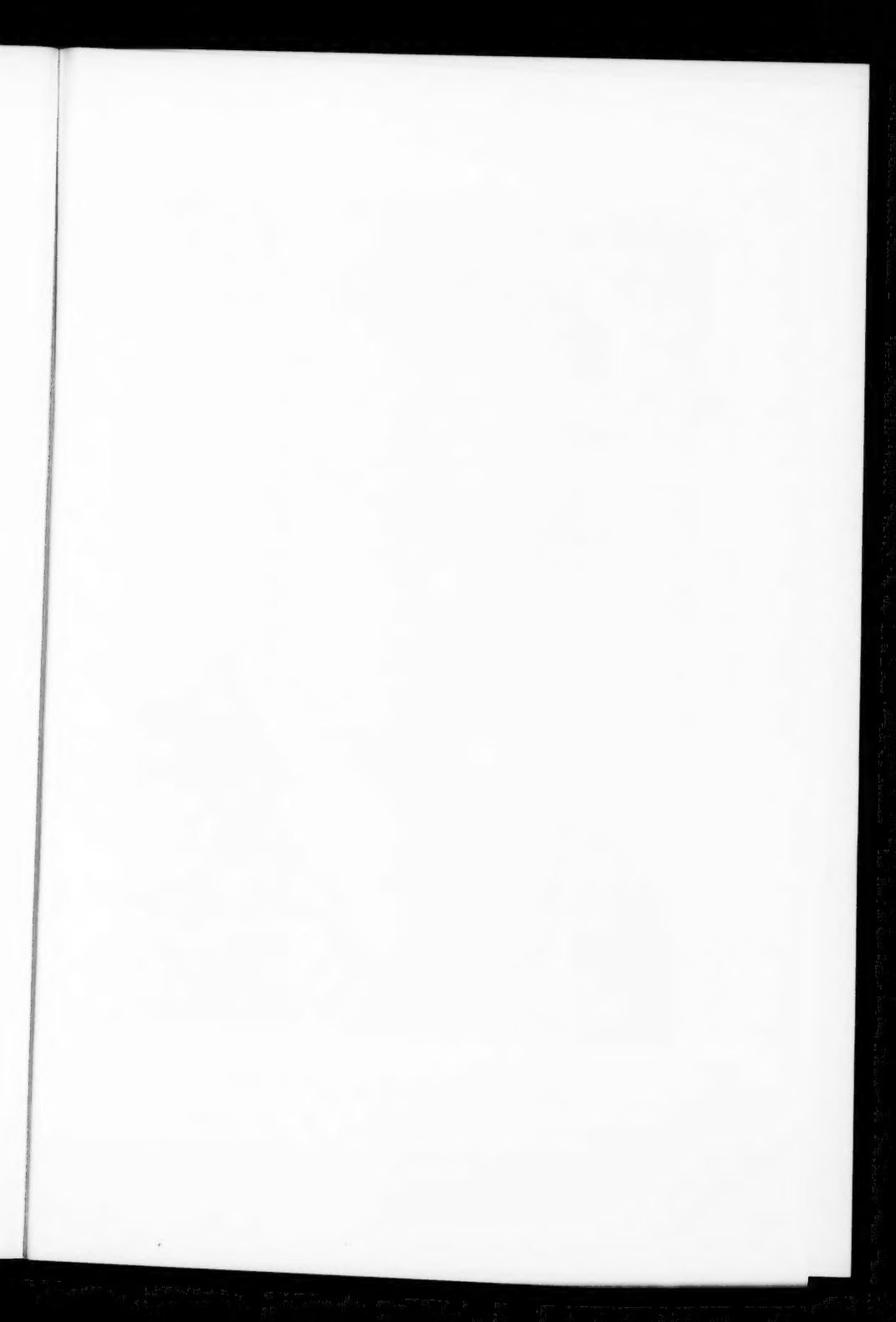
We have enjoyed the devoted help of Jean Armstrong as editorial assistant in preparing this volume for the press, and of Richard Wold, who did the subject indexing, and Frances Yost, our author indexer. Our thanks should also be expressed to the George Banta Co., our printers for many years. At various times the full editorial staff of Annual Reviews has come to our help, and the business staff, though serving in anonymity, has played an indispensable role.

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F.W.A.	G.M.
H.E.C.	E.E.S.
J.M.L.	E.S.

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Wm Mansfield Clark

NOTES ON A HALF-CENTURY OF RESEARCH, TEACHING, AND ADMINISTRATION

BY WM. MANSFIELD CLARK

Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland

*When I find a bit of leisure I trifle with my papers. This is
one of the lesser frailties. Horace, Satires I, iv.*

A LITTLE OF THE BACKGROUND

The only wisdom I've displayed was in the choice of ancestors, parents, teachers, chiefs, and associates. Persons in each category played decisive roles in whatever may have led to the flattering invitation to write this sketch. Therefore, biographical notes, which alone have no interest in this case, are used to illustrate the importance of human relations in molding a career. It is very embarrassing that space is inadequate for mention of all participants and for full appreciation of those mentioned.

I chose my ancestors for their rugged constitutions. Such a constitution makes enjoyable sustained, hard work. Hard work is essential as a way of getting on, if a scientist is not brilliant. Therefore, I advise any scientist of this sort to choose his ancestors as I did and to remember what the Greeks said: "Before the gate of Excellence the gods have placed sweat."

The unborn scientist looked for parents having superior characters and found them. Later he discovered that one was a clergyman in the Protestant Episcopal Church and the other a daughter of another clergyman of the same cloth. I have not had the nerve to wear in an academic procession my father's hood, given him by his Alma Mater, Columbia, with the honorary degree S.T.D. (Sanctae Theologiae Doctoris), but I have the itch to see if this monstrous deception would be detected.

James Starr Clark was born in Brookfield, Conn. in 1822. His first wife died at an early age without leaving a child. At the age of 51 he married Caroline Hopson of Vermont. Their third child and only son was born in 1884. These circumstances gave the boy a father who was a mature man of wide experience. If but one item of the mother's experiences were to be selected as pertinent to what follows, it might be this: She learned in a hard way the poignancy of Sir Roger de Coverley's remark "Much might be said on both sides." One brother fought on the Union side and was killed in battle. The eldest brother, having gone from Vermont to Georgia, made a hard decision and joined the Confederate Army. Mother named her son William for the beloved rebel and Mansfield for her ancestor Bishop Richard Mansfield. At mention of this the Bishop's bones must shudder.

Father's chief interest beyond the Church was in education. He discussed with Mr. John Bard the founding of St. Stephen's College, now called Bard College. When he built his church with Mr. Bard's support, in a district which then had no public school, he appropriately put school rooms on the

ground floor and the chapel on the upper floor. In 1867 he established Trinity School for boys in the township of Tivoli, N. Y. The school flourished until the financial panic of 1893 which led to the withdrawal of students and then to the foreclosure of mortgages on the church building, which Father had assumed, and on the many improvements at the School.

On his school property Father had introduced some good farm practices to encourage better farming in the district as well as to provide healthful food for his boys. He had a keen interest in sports. In each of these departments he introduced some features sufficiently unique to be called experimental. Thus it may be that his son inherited a penchant for experimentation. However, in the son this began in a bizarre manner. Examples: His wood-fired, stone furnace failed to melt "tin" cans, a project conceived to supplement a store of tin foil as a source of wealth. He tested the ability of a muzzle-loader to withstand shock when fired with the barrel half full of gunpowder and half full of nails. The gun survived, but William had not noticed that the gun, strapped to a box, was pointed at the chicken house at roosting time. That the little devil was not punished attests his father's own interest in experimentation as well as his father's amusement in the miracle of no chicken killed, although a few were somewhat defeathered.

There fell into the boy's hands Tyndall's famous lectures on sound and light and George M. Hopkins' *Experimental Science* (1890). The latter was especially entrancing because many experiments could be repeated without elaborate equipment. Having no scientific instruments whatever the youngster acquired the habit of saving junk with which to make gadgets, a habit that amused associates in later years.

When boyhood began to be left behind, the playful interest in science merged with a serious concern. This was accompanied by a change of outlook. The details of this gradual change have no present interest but I boldly suggest that the following note may have some interest because it concerns a transduction of attitude somewhat typical among persons of my generation.

Of course I cannot describe my parents' thoughts; what was shown by their attitude made an enduring impression. Abiding in a great tradition, they appeared to accept its doctrines as a matter of course. These I never heard discussed at home. They practiced Christian ethics so well that they are remembered with deep affection by all who knew them. Also, their attitude toward others had that delightful tang of New England in its finest period: Anyone could be as radical, or eccentric, as he pleased, provided he behaved himself—and occasionally went to church. Because of Father's long experience with boys he would have said that rebelliousness, like measles, should be contracted early, the first to induce an immunity against snap judgments. So it was that when I leaned definitely toward science in contemplating the controversies that were still raging in the post-Darwin era, Father did not argue. In the serenity of his own convictions he instilled the precepts that understanding and appreciation of any major matter can be attained only by earnest, scholarly effort and that firm conviction is established only by the exercise of such effort in complete freedom. Withal there

must be goodwill and integrity. The latter were the chief concern of Mother and one must try not to let her down.

But suppose that in following these precepts one were to attain the attitude expressed by the quotation: "Do not ask, by chance, what leader I follow or what godhead guards me. I am not bound to revere the word of any particular master" (Horace, *Epistle* 1,1).

If followed without discipline, this could lead to disagreeable ends. However, they were men of cultural disciplines who founded the Royal Society and who took from the cited passage in Horace *Nullius in Verba* as the motto of the Society. It would appear that they found the original text inadequate to express full implication of the motto, for the very free translation, here quoted, is that preferred by the Royal Society—so Dr. Henry Allen Moe informs me. Dr. Moe suggests [*Science*, 132, 1817 (1960)] that in 1660 adoption of this motto signified only a breakaway from Aristotelian authoritarianism. Today a much broader base may be found. Not only have very many new phenomena been revealed by experiments; many a genius has intuitively predicted what had not been conceived and has been confirmed abundantly by experiments. The almost indefinable complex of experimentation and intuition, expressed briefly by "the scientific method," has been so successful as to have created a strong, emotional appeal, not alone because of material benefits, but largely because of the grandeur of expanding knowledge. We bask in the delights, know the zest of adventure, feel the warmth of comradeship. This is not to say that the more subtle parts of science grow less perplexing. Scientists go blithely on their way creating more puzzles than philosophers seem able to solve decisively. This is part of the excitement, the origin of a profound modesty, and the basis of what those who have been cultured in more dogmatic traditions find difficult to understand: that the sportsmanship of the pursuit is worth more than the quarry. For no theory is sacrosanct; one used today may be revised tomorrow. Some theories concern different dimensions—a matter put tersely by Robert Millikan: "Einstein must include Newton."

Scientists are no more virtuous than others but virtues inherent in their traditions come to the fore. The faking of data is the unforgivable sin. The twisting of interpretation to conform to wishful thinking is not much less. This practice has been called "virtuous lying" when it suits a noble purpose. A danger in wishful thinking is that it may destroy the basis of free and profitable inquiry and thus give to tolerance a dismal emptiness. In a company of scientists, the slightest trace of wishful thinking is greeted with chuckles. Unfortunately, the raucous laughter of the snobbish "wise guy" sometimes obscures a delicacy. When a true scientist chuckles over a slip it is partly at himself for having also thought wishfully, as do all human beings. When one detects in this honesty, modesty, and generosity one wishes that he himself and all men could have so clean a mind.

Scientists have no monopoly of the objective attitude, but their easier and therefore more frequent use of it may set patterns of conduct for a distraught world in its attempts to solve some extremely complex problems—

problems for which science itself has now no solutions. Although one may not contribute substantially to all this, he may be proud to do what he can.

Obviously these remarks are colored by what has come with age, but they reflect the impact of science upon one youngster. So it might be said that those genes which determined the attitude of the parents in their intellectual efforts, rather than what the *Zeitgeist* had contributed to the subject matter of their thought, took command of the son. Much the same in different forms may have been, and may yet be, true from generation to generation. For the present, much of the hold-over may well be left to that blessed silence which is the privilege of free men who know that they cannot escape entirely from the traditions of their era. Some would not escape, if they could, so long as they find in these traditions the urges that stir the artist and the poet in ways that never will be defined precisely, yet somehow preserve and create the beauties which give to life its values. It is this that makes the humanities as important as the sciences and all coordinate in their civilizing effects.

ON A FEW TEACHERS

During the moves that followed the abandonment of Father's school, my education in public schools was humdrum until I was fortunate in being admitted to Hotchkiss School to prepare for college.

The Master of English at Hotchkiss, Mr. Buehler, took an interest in the country bumpkin. This may have been because the youngster displayed some feeling for the King's English, which had been cultivated at home, and because of a very remarkable originality in spelling. At Williams College, Professor Maxey illuminated the youngster's essays with generous amounts of red ink. The coaching given by these teachers led first to the editorship of the *Hotchkiss Record* and then to membership on the board of the *Williams Literary Monthly*. The pleasure attained in writing, as a fulfillment of an inherited urge to teach, has compensated for the labor. I doubt that I have written anything without at least three or four revisions; often many more.

The Master of Mathematics at Hotchkiss was "Uncle Joe" Estill, who was universally loved. In later years he was my delightful companion in golf, and in his 80's was a master of the "brassie approach" with which he often won from the long-hitters. This genial but hard taskmaster commanded attention without one knowing how he did. I quickly gained the impression that I had better do well in math.—or else—and that it would be worth while to try. The plan which I devised was this: Catch on to the manner of thought by studying a few propositions and their proofs in the text on plane geometry. Then, without looking at the proofs, contemplate other propositions until a proof for each is devised. The self reliance thus established together with good instruction made mathematics enjoyable in school and college. It is unnecessary to say that I acquired no real ability in this field. The trivial experiment is mentioned because it now appears to have been the origin of a tendency to restate scientific matters in my own way. In teaching I have found that students are individualistic in their studies of any scientific subject. Some quickly grasp the meaning of a cryptic statement. Others need the

matter to be spelled out and even then what an author, or a lecturer, believes to be a clear statement may contain some quirk that throws one or another student off the track. I like to believe that, despite obvious limitations, my constant efforts to state matters in my own way so that they are clear to me may have helped those who happen to have limitations similar to mine. Some generous people have said that they have been helped by my books.

To return for a moment to mathematics; it may be noted that the old way of teaching some of the subjects was chiefly by testing the ability of a student to follow the book and to insert properly in a general formula the numerical values given in specific problems. Irving Langmuir told me that the most valuable course he had taken was a survey of the different sorts of mathematical thought. We had none of this.

In French and German, as in Latin, we "got by" with halting translations and then were subjected to drills in grammar. This was so dreadfully boring that I neglected the language courses, thus establishing a handicap in the scientific career.

Being concerned now with subjects bearing more directly on a scientific career, I omit mention of several inspiring courses among those which made Williams College outstanding as a liberal arts college.

Dr. Leverett Mears, Professor of Chemistry at Williams, introduced his subject with a little book of questions and answers. Finding this quite uninteresting, I read Remsen's text instead and got caught on the first recitation. This led to some kindly, special attention by the Professor. What was lacking in his primitive text was more than compensated by his lectures and lecture demonstrations. These made the subject exciting. I liked chemistry. On Professor Mears' recommendation I was made the teacher of chemistry in the Williamstown high school in my senior year.

During the class banquet after graduation I received a note requesting me to see Professor Mears in the morning. At that interview he asked me to be his assistant, guaranteed time for advanced studies, and said that he would recommend me for admittance to the Johns Hopkins University as a graduate student. I was then engaged to teach in a preparatory school but when the offer was explained to the headmaster he graciously relieved me of my obligation. Thus, the kindness of two men led to a sudden change in the direction of my career. After another year at Williams I went to Hopkins with a letter of introduction to Professor Harmon Morse from his old friend Mears.

As is well known, the Johns Hopkins University, under the leadership of President Gilman, had inaugurated a profound change in American universities. The emphasis was on original investigations. This is of great importance. But let it not be forgotten that other functions of a university include the conservation of established knowledge, its promulgation with the new, and some guidance of immature students. In my particular case an imbalance became evident but I was too timid to get on balance myself. I became the student assistant of Professor Morse who, to keep my services, had me finish only a small part of his course in quantitative analysis and bargained to have me relieved from all work in the laboratory of organic chemistry and

from more than a couple of experiments in the laboratory of physical chemistry. At that time the major technical difficulties in exact measurements of osmotic pressure had been overcome and the measurements, aimed to document a principle, had become routine. There remained much to take care of and, although the care was shared by several people, I was made responsible for a great deal. This I liked and became the willing and worshipful slave of the finest experimenter I have known.

Morse was a very dull lecturer. On one occasion he described an apparatus that included a Bunsen burner. He drew all parts of the burner including the zones of the flame. Drawing provided the opportunity to drop the eraser, in bending for which he got rid of tobacco juice. I saw in that sketch of a Bunsen burner more than met the eye. Morse must have in mind all parts of an apparatus so that no detail might escape his thoughtful attention. I cannot be too thankful for having had this drilled into me, and when I recall how often it has stood me in good stead I forgive the grand old man for his having diverted me from some parts of experimental chemistry. Indeed the training he gave me may be said to have been justified in that when I undertook independent investigations there was no hesitation in making a direct attack, if not with theoretical insight, at least with apparatus that I understood and, if necessary, made myself.

There are many stories of Morse's care for detail. One will suffice. One day I found him checking calculations. He was multiplying and dividing the long way. I said "Professor, why don't you use logs?" He replied: "There might be an error in the Tables."

CHIEFS AND JOBS

During the student years I spent several summers at Woods Hole, where my brother-in-law, Dr. Francis B. Sumner, was in charge of the local station of the U. S. Fish Commission. At first I worked with the crew that collected marine organisms. Then a chemical laboratory was established in the Station and put under Dr. Carl Alsberg of Harvard. I was made his only assistant. In a book written by several distinguished persons to honor Dr. Alsberg, Dr. D. D. Van Slyke quotes from a letter found among Alsberg's papers after his death. I quote that passage from my letter to show part of my indebtedness to Carl Alsberg.

"I wonder if you remember a hot night at Woods Hole when I confessed to you that the Fish Commission had given you a mighty ignorant assistant and you took me to your room to tell me about the beginnings of biochemistry and how Spallanzani discovered pepsin by the mean trick of tying a string to a bait and deluding a duck. When you play with your grandchildren and fame has lost some of its glamor, please recall that night as one on which you started a kid on his ambition to learn the heritage left to us by Spallanzani, Bernard and Pasteur."

Later we renewed our acquaintance in Washington, where his recommendation had brought me and where he was first in the Bureau of Plant Industry and then Chief of the Bureau of Chemistry. Later he became one of the directors of the Food Research Institute at Stanford to which position he

brought, in the words of Robert Calkins, his "easy-going manner, wide acquaintanceship, quick grasp of ideas, roving interests, deep wisdom, broad knowledge, understanding and tact."

Dr. Alsberg having entered the Government service, Dr. D. D. Van Slyke was put in charge of the chemical laboratory at the Station the next summer. On the way to Woods Hole he became seriously ill and was taken to a hospital in Boston. During periods when he verged on the delirious he imagined all sorts of things, and fortunately he remembered later that nitrous acid had liberated nitrogen from an amino acid and that he had stuffed the nitrogen into a eudiometer. This was the origin of the neat device with which Van Slyke determined amino nitrogen in solutions of hydrolyzed proteins and, with supplementary methods, distinguished groups of amino acids. To appreciate the value of these methods one should know the period. The device may also be said to have been the forerunner of the more famous apparatus with which Van Slyke developed gasometric methods of analysis in wide variety. He is an ingenious analyst, but far more. Each of his methods has been designed for an attack on a fundamental problem in biochemistry or physiology and for his magnificent achievements we all revere him. He won my affection by treating me as if I were his collaborator instead of the laboratory boy that I was.

Being left alone during Van Slyke's illness I did some work which is amusing when related to what is found in my first book and which was destined to have a consequence to be mentioned later. Assuming that my chief would require standard solutions, I had the notion that a solution made with redistilled ammonia would be better than one made with sodium hydroxide, which usually is contaminated with carbonate. To be sure of the titer I used every indicator I could find at the Station, including some histological staining agents which I begged from Leo Loeb. Great was my disgust to find that hardly two of these indicated the same equivalence point in the standardization of the ammonia solution.

Suggestive of the state of the subject at that period is the fact that at Hopkins there had developed a controversy between one professor who emphasized Ostwald's simple theory of indicators and another professor who emphasized the tautomeric changes. Although one or the other professor may have related a theory of indicators to the theory of titration, I never heard this discussed. When I was asked a question on phenolphthalein on my final oral examination, the question was couched in such a manner as to cause a renewal of the controversy. The two professors wrangled so long that I was spared the humiliation of revealing my ignorance of many matters.

Near the close of my graduate work, Dr. Alsberg had recommended me to Mr. Lore Rogers who was in charge of the research laboratories in the Dairy Division, U. S. Department of Agriculture. Mr. Rogers engaged me. This was great good fortune. When I reported on July 1, 1910 I asked Mr. Rogers what I was supposed to do. He replied "That is for you to find out." He meant this literally. He left me to my own devices and never showed impatience with my

initial floundering. Risky as was this policy, it provided the opportunity for the initiation of my major lines of research. Rogers not only provided new equipment and help as the need arose; more important, he endured my somewhat rambunctious expressions of opinion without losing confidence in the work on which the opinions were based. This was an invaluable lesson in objectivity.

After ten happy years in the Dairy Division, and with considerable regret in leaving Mr. Rogers, I accepted the opportunity to expand my work as Professor of Chemistry in the Hygienic Laboratory of the U. S. Public Health Service. The Hygienic Laboratory was the precursor of the National Institutes of Health.

The professors were regarded as academic adjuncts to an essentially military organization. They were supposed to follow their own inclinations in research and that the professors of chemistry did so is made evident by the variety of their principal interests. The first Professor of Chemistry, J. H. Kastle, was an authority on oxidases; the second, E. C. Franklin, pursued his famous studies on liquid ammonia solutions; the third, E. B. Phelps, was a leader in the study of the pollution of water supplies; my interest was chiefly in measurements of oxidation-reduction potentials and their systematic formulation; the fifth Professor, C. S. Hudson, continued at the Hygienic Laboratory, and subsequently at one of the Institutes of Health, his classical studies of carbohydrates.

Later, I shall mention contributions and contributors to my special field in the Division of Chemistry between 1920 and 1927. Space is inadequate for comments on several other investigations. Among them was a study of the clarification of water supplies by aluminum sulfate and a study of the distribution of lead in persons exposed to lead tetraethyl. An accident at a plant where lead tetraethyl was made had caused such distress that this substance came to be known as "loony gas" and experts in industrial hygiene recommended that its use in gasoline be not permitted. Space also is inadequate for comment on all members of the staff. I'm sure they will not be offended by special mention of Barnett Cohen. This Yale student was permitted to complete his dissertation for the Ph.D. degree in the Division of Chemistry. We worked together for many years in Washington and Baltimore. The genial, learned and modest "Barney" had a stabilizing influence on me and on two departments which I acknowledged in the memorial article which I wrote for *Bacteriological Reviews*, a journal which Cohen edited for 15 years. He and I became presidents of the Society of American Bacteriologists, a rather rare occurrence for contemporaries in the same laboratory. Cohen was a learned bacteriologist as well as a fine chemist. I got in by a back door.

From time to time I was asked to take charge of one or another department in a university. The usual basis for declining was that I had had no formal training in any of the subjects I was supposed to "profess." This was made to appear of secondary importance in a very remarkable action. In the Spring of 1927, without any preliminary hint of which I was aware, Dean

Weed informed me that I had been appointed De Lamar Professor of Physiological Chemistry in the School of Medicine, Johns Hopkins University. During a visit, I remarked to dear old Professor Abel that I could not see how I would fit in the School of Medicine inasmuch as I did not know, for example, the difference between the thymus and the thyroid glands and less of their functions. Professor Abel replied: "My dear boy, that is why we want you." This exaggeration could be interpreted only as meaning that the School followed the Hopkins tradition in encouraging research without regard for immediate applications, an impression re-enforced by all those with whom I talked. The whole affair was conducted in such a way as to make me feel that this was one of those times when a person must defer to the judgment of others. The challenge, thus presented with assurance of confidence in my ability to meet it, and a yearning for the atmosphere of the university constituted a compulsion to accept.

I had had no formal training in biochemistry, had an inadequate appreciation of the needs of medical students, and inherited laboratory equipment and space totally deficient in what was required for my research and student instruction. I needed support desperately. It was given effectively. Funds were provided for equipment needed immediately, I was allowed to modify plans for a new building, and then everyone left me completely alone as a token of confidence. The most important part was that "everyone" constituted a company of gentlemen and scholars who took it for granted that each of their company would do his best to improve his best. It was this that made the autonomy of the departments so successful and the atmosphere incomparably congenial and stimulating.

After 25 years as Professor of Physiological Chemistry I was made Research Professor of Chemistry, largely by the action of former President Bronk with the approval of the Academic Council. The Department of Chemistry provided a laboratory, and my successor, the brilliant and charming "Al" Lehninger, let me take much of the special equipment I had used. Having been the beneficiary of these and many other generous acts I have a warm place in my heart for good old Hopkins. After some unprofitable investigations in which I felt free to indulge I devoted my major effort to compiling data and writing chapters for *Oxidation-Reduction Potentials of Organic Systems*, a book which I felt was needed.

A FEW NOTES ON TEACHING

A few years ago Vincent du Vigneaud reminded me that while he was with Abel he audited my first lectures to the students. I remarked "Those were doubtless the worst lectures ever given in biochemistry." With a twinkle in his eye "Dee" said "Those on pH were well presented." I could add only "Touché!"

In the order of appointment, Mary Buell (who, to my delight, remained after Professor Jones retired), Leslie Hellerman, Barnett Cohen, Eric Ball, Logan Irvin, Francis Chinard, and others cooperated in making the course

what I am sure became a very good one. I shall have to call upon those who know the scientific achievements and personalities of the persons named to recognize that each of them gave superb instruction.

During the earlier part of a quarter-century of teaching I would have guessed that an individual medical student placed the required course in physiological chemistry at one or another point between a disciplinary hurdle and a cultural course having some promise, but little immediate use, in the practice of medicine. Over the years a noteworthy change in attitude occurred, but at first we had to contend against a lack of incentive to learn on the part of many students, although, as a class, the students inspired their teachers by the earnestness of their efforts.

In the course of these 25 years a second difficulty was brought forcibly to our attention. During these years the students came from nearly 200 different institutions, among many of which the standards were very different. We all agreed that to provide a basis on which the students could build we should pick up the threads of thought in basic chemistry before weaving them into biochemistry. But with the entering students differing widely in attainments one hardly knew where to begin.

No one desires educational programs to be uniform for the sake of uniformity, but surely there are some principles of chemistry that permeate the whole science. In the 30's it was estimated that about half of the entering students had had inadequate instruction in those more elementary parts of physical chemistry that are involved in almost all parts of the science and that are essential in a first approach to an understanding of matters of clinical importance. To meet this particular requirement I wrote *Topics In Physical Chemistry* as a book to be consulted according to the need of the individual. To call attention to the broader problem I gave the address *A Challenge to Scholarship* [University of Pennsylvania Bicentennial Conference, University of Pennsylvania Press (1941)]. Some parts of this address now sound like post-Sputnik complaints of our educational system but these parts led to what has been overlooked by many authors. To analyze an exact science so that one may give the greater emphasis to principles that penetrate the deeper, and that give order to the greater number of facts, requires a comprehensive knowledge and great good judgment in its use. To avoid the new dogmatism, exemplified by the elementary text that contains little more than statements of conclusions, it is essential that instruction include at least typical items of evidence and descriptions of the means by which the evidence was accumulated. All should be presented with brilliant clarity and without abandonment of the essentials of rigorous reasoning in theoretical matters. All these factors constitute an intellectual task of high order and great difficulty. It may be presumptuous to assume that scholars in universities are the better equipped for the task, but the assumption is a fair one. However, by and large the atmosphere has been more conducive to "research" than to re-search. Had a more artistic division of interests prevailed, the results of efforts in the direction indicated would inevitably have trickled down the ladder up which the student climbs. Thus it may be said that the

responsibility for complaints, which at long last have become strident, rests in no small measure with the universities. Included among their duties is that of easing the way of students to attainments greater than those of the teachers. Thoughtful easement of the acquirement of knowledge is an essential part of the economics and of the aesthetics of education. It should not be regarded as less than among the tougher of intellectual jobs. A department that leaves instruction in the basic parts of a science to persons of low rank, no verve, and little appreciation of the magnitude of the task contributes stupidly to a dull future.

A FEW OF THOSE INVOLVED IN THE EVOLUTION OF RESEARCH

Early in my work in the Dairy Division I studied the two types of holes in Swiss cheese; one type ruinous, the other typical. A nice old lady in my home town used to plague me periodically by the request: "Do tell us how you put the holes in Swiss cheese." This little investigation is mentioned because of a consequence. To meet the experimental requirements, I had managed, without previous experience in working with glass, to build a mercury pump and to devise special means of isolating and analyzing gases over mercury. I was prepared to help Mr. Rogers when he asked me to suggest to him a technique for the study of the production of gases by bacteria that would be better than the use of the old Smith tube. He was then engaged in taxonomic studies of the "colon-aerogenes" group of bacteria. With some assistance by Brooke Davis and Alice Evans, who became distinguished especially for her work on brucellosis, we found in a very large number of experiments distinct differences in the ratio, volume of CO_2 /volume H_2 , in the gas produced from glucose. This became the basis of Rogers' classification. Later, Lubs and I found that our simple "methyl red test" correlated well with the gas ratios and other differential tests. This was my introduction to bacteriology.

Several practices were puzzling. For example, what was the significance of the large differences of "acidities" among different culture media and cultures as determined by titrations with phenolphthalein the indicator? I recalled the trouble with that ammonia solution, previously mentioned, and that Van Slyke had suggested that an article by Salm might illuminate the difficulty. Without Van Slyke's suggestion I might have plodded along an old road. I looked up Salm's papers and, in further study of the literature, ran across Sørensen's famous paper published in 1909. This was so illuminating and inspiring that I promptly designed my own hydrogen electrode outfit and began work on several problems that need not be reviewed here.

Today, when the principles of acid-base equilibria are widely known, persons of this generation may have difficulty in appreciating the former state of the subject in the biological sciences and in understanding why anyone should have taken the trouble to expose fallacies which now are as plain as a pikestaff. For example, as determined by titration with phenolphthalein the indicator, cows' milk seemed to be much more acidic than women's milk. Accordingly some pediatricians recommended the addition of lime water, or

milk of magnesia, to cows' milk when used for infant feeding. As a matter of fact, both sorts of milk have about the same pH number, near 7. My critical paper on this subject led to the abandonment of the use of lime water, etc. for the purpose of "neutralizing" the "acidity" of cows' milk for infant feeding. When I was given a Borden Award, this was doubtless referred to in that part of the citation which the President of the American Chemical Society read to the members as "a contribution to infant breeding" (sic).

Shortly after the work with the hydrogen electrode was begun, Mr. Rogers engaged Dr. Herbert Lubs to be my assistant. Lubs is an enthusiast who enlarges a suggestion. One day I said to him: "Let's assemble all the indicators we can find and extend Sørensen's work systematically." Said Lubs: "Why fool with old indicators. Let's make some new ones." So Lubs synthesized several new members of the sulfonphthalein series and I did the physicochemical work of characterizing each. These indicators are sufficiently brilliant to serve well in approximate measurements of the pH numbers of colored and turbid culture media and cultures. By concurrent measurements with the indicators and the hydrogen electrode we demonstrated the usefulness of the indicators in attacking several problems in bacteriology. A paper thereon was published in the *Journal of Bacteriology* in 1917. At that time bacteriologists, many of whom had had little advanced training in chemistry, were eager for such help as chemists could give. To this circumstance may be attributed the rapid exhaustion of the numbers of the *Journal* in which our paper had appeared. Then Mr. Charles Thomas, representing Williams and Wilkins Company, suggested that the paper be reprinted as a separate. I may have said something like: Shucks. The subject is broader than what was given in that paper. How about a book? This, in brief, was the origin of *The Determination of Hydrogen Ions*, the first venture of Williams and Wilkins in the publication of books. The first edition was that of 1920; the third and last edition was published in 1928.

This book put in convenient form what anyone could have found in the literature if he took the trouble to search. Much had been given in Michaelis' excellent *Die Wasserstoffionenkonzentration* (1914). It may be fair to say that the appeal of my book was its coordination of elementary theory, the techniques with which the theory can be applied, and sketches of some of the innumerable applications. The book was essentially educational. Recalling the remark that if an author steals one statement he is called a plagiarist but if he reconstructs many statements and appends an impressive bibliography he may be regarded as a scholar, I have been somewhat embarrassed by the mention of this book as the chief item in citations accompanying some honors. I mention honors egotistically, but also to seize the opportunity to say that on each of three occasions Baird Hastings introduced me with such wit as to bring down the house. I treasure some of his remarks despite the distortion of his scientific judgment by his heart. Such generosity is an aspect of scientific life that should not be left unnoted.

To Louis Gillespie must be attributed the stimulus for a broadening of the field of research. Gillespie had a remarkable career, from early work in

bacteriology to work in the field of thermodynamics at M.I.T. When I first knew him he often came from the Bureau of Soils to my laboratory to discuss scientific matters in general and his own problems in particular. One set of experiments fascinated me. He had constructed an electric cell with a calomel half-cell and an electrode of pure mercury overlaid with a culture of bacteria. As the culture developed, the mercury electrode became progressively more negative. This he interpreted to mean that the bacteria were producing an intense reducing environment and he foresaw possible consequences of such an environment under the anaerobic conditions of water-logged soils. It occurred to me that Gillespie had an experimental basis for investigating the differential reduction of dyes which bacteriologists had noticed in cultures. After repeating Gillespie's experiments in various ways, I made the more definitive studies of the oxidation-reduction potentials of the methylene blue system and of a sulfonate of indigo system, published in 1920.

At that time I was not aware of Haber & Russ' preliminary study of the benzoquinone system, published in 1904, or that Biilmann in Copenhagen and Granger in Nelson's laboratory in Columbia were studying the benzoquinone system. Biilmann's and Granger's papers were published also in 1920. At about the same time Jim Conant told me that he had planned potentiometric studies of quinones. His studies and those of Miss Baker in LaMer's laboratory were soon published. All this is a striking example of what comes with the "ripening of time." Curiously, none of those who studied organic oxidation-reduction systems prior to 1920, with the partial exception of Haber & Russ, had provided conditions for stable and definitive potentials. In my case the use of buffer solutions had become routine and, as a matter of course, I titrated methylene blue, for example, in each of several buffer solutions and thus stumbled on a series of finite ratios of the concentrations of particular species of oxidant and reductant at each of a series of pH numbers. As the data for methylene blue accumulated, they lined up beautifully on a diagram like nothing seen before. I was elated. I recall saying to myself: "This general subject will keep me busy for years." It did. When the range of compounds was extended, the range of the values of ionization constants was such as to provide a variety of good illustrations of the integration of states of equilibria in exchanges of electrons and protons.

A preliminary survey of some of these systems was made in the Dairy Division with some help by H. F. Zoller, and at the Hygienic Laboratory the work was made more systematic with contributions by Barnett Cohen, M. X. Sullivan, H. D. Gibbs, R. K. Cannan (then from England), Max Phillips, W. L. Hall, and Paul Preisler, who came after I had gone to Hopkins. The studies were continued at Hopkins with contributions by my students. R. D. Stiehler, T. T. Chen, H. J. Lowe, Henry Harbury, my assistant Miss Marie Perkins, and by Dr. Eric G. Ball.

In all of our early work, and within the conditions which we happened to use, the data conformed so well with the postulate of a simultaneous two-equivalents change as to give the impression that in organic systems this predominates, except in rare cases. Although by 1925 we were well aware of

Conant's potentiometric study of free radicals of the triphenyl-methyl type we failed entirely to look for free radicals in the systems which we studied. In one or two cases in which we might have picked up evidence, the small departures of our data from the form of the titration curve postulated were ascribed to our experimental errors or to the ever-present danger of having impure substances.

This reminds me of a remark by Professor A. P. Mathews. We found ourselves on the same platform. After I had presented some quantitative data, Mathews prefaced his address with the remark: "Clark is so interested in the fourth decimal place that he never made a discovery." At lunch with this charming character I got back at him by kidding him about his "mentets," units of a mental force analogous to well known forces.

In my book, *Oxidation-Reduction Potentials of Organic Systems*, is a brief account of how the favorable properties of pyocyanine led Cannan, and later Elema (1931) and Friedheim & Michaelis (1931), to consider its step-wise titration curves. In the published papers, the steps were interpreted to mean the formation of an intermediate free radical, or "semiquinone," as Michaelis called it. Michaelis and his associates pursued this subject extensively and brilliantly for many years, revealing by several methods the formation of intermediate free radicals in a large number of cases. Usually the free radical forms the more extensively at very low, or very high, pH.

After the hectic period during which my Department at Hopkins was re-organized, a new building constructed and new apparatus installed, Leslie Hellerman called my attention to a matter which led to another dimension in the subject of my research. Evidences were accumulating that a coenzyme and a specific protein are jointly concerned in one or another biological oxidation-reduction process. As a matter of course I asked this question: If the coenzyme is subject to oxidation-reduction, what will the effect be on the potentials of a different firmness of bondings between protein and reduced coenzyme and between protein and oxidized coenzyme? It seemed impractical to study the natural systems which then were in crude states and with our techniques as they were then developed. An answer to the question had been given in principle by Conant's initial study of the heme system in the presence of one or another ligand. I undertook further studies of metalloporphyrin-ligand systems, both for their intrinsic interest and to illuminate the broader problem. At about the same time Barron & Hogness, with their associates at the University of Chicago, started their studies of metalloporphyrin-ligand systems.

Notable contributions were made by my students: John Taylor, Harrison Davies, Carl Vestling, James Weisiger, Curt Porter, R. Lewis, R. W. Cowgill, L. L. Rosenberg, H. A. Harbury, Miss Marie Perkins, and Dr. Joseph Shack, a National Research Council Fellow.

The more we studied these systems the more complicated they were found to be. Indeed, it is impractical to review the subject here. Two aspects may be noted: (a) The limitations of particular experimental methods were striking.

This required an immense amount of work to attain a consistent set of formulations. No claim is made that they are final. (b) When all conditions are constant except the concentration of a ligand, and at a given degree of reduction, there is a shift of potential to more positive values with increase of ligand if the bonding between ligand and reduced metalloporphyrin is firmer than that between ligand and oxidized metalloporphyrin, and a shift in the opposite direction if the bonding between ligand and oxidized metalloporphyrin is firmer than that between ligand and reduced metalloporphyrin. We have cases of both sorts complicated by changes of states of aggregation, changes of pH, etc.

Although in our cases additions of ligands are reversible, I have shown in *Oxidation-Reduction Potentials of Organic Systems* that the same principle should apply in cases in which the additions of ligands are practically irreversible. Accordingly, the principle may be invoked to account for the great differences between the potentials characteristic of conjugated proteins containing the same metalloporphyrin, and between those characteristic of flavoproteins. Theorell & Bonnichsen used the principle in their discussion of one of the alcohol dehydrogenases. The possible applications are many and they have not yet been extensively explored. The relation to the biological problem involves this question: Are the large differences of potential, which are characteristic of these systems, important *per se* or are they the fortuitous results of the natural selection of other properties suited to the unique function of each of these systems?

On several occasions I have used data for acid-base equilibria, oxidation-reduction equilibria, and equilibria involving ligands to illustrate a "chemical continuum." In a system involving these somewhat arbitrary categories the importance of one constituent may decline while that of another increases with a change of conditions. Large changes of electrode potential may indicate an enormous change of the ratio of concentrations (or activities) of conjugated constituents. The smooth continuity of the potentials impresses the observer with the smooth continuity of the material changes. This view is not new; our data provide good illustrations, several of which are given in *Oxidation-Reduction Potentials of Organic Systems*.

To illustrate a matter of general interest, it is unnecessary to say more than that in the simplest case, one involving only exchanges of electrons and protons, relations may be described by a surface in three dimensions and that there are two aspects of such a surface. By control of conditions, one may determine the unique contours of such a surface. This done, one has a view of a surface over which there could be an infinite number of possible paths of change. A chemical continuum so described is ridiculously primitive compared with one which may be conceived for a living cell. Only to indulge in a bit of elementary philosophy, let us make the strange assumption that so simple a system were to be segregated in some one phase of a living cell. By studying the isolated system under controlled laboratory conditions one could provide a description of the sort already noted. Only experiments *in*

situ could show what particular path, or trend, is followed under natural conditions. Knowledge of this might provide no hint of why the unique path, or trend, is followed. These are three aspects. To describe is one thing; to explain in terms of cause and effect may be a very different matter.

EDITORIAL WORK

While a member of the Editorial Committee in 1933, I was shanghaied by Stanley Benedict and put to hard labor on the Editorial Board of the *Journal of Biological Chemistry*. I served for about 18 years under Benedict, Van Slyke, and Anderson, all very wise and efficient managing editors.

There are so many aspects of editorial work that I must confine attention to two. Because the substance of a scientific paper is of supreme importance there is ample room for differences of opinion on the importance of its literary quality. A member of one board said that he would accept a paper for the value of its substance without any regard for its literary quality. I held the following view. The greater the scientific value of a paper the greater the probable number of man-hours devoted to its study and the greater the obligation of the author to see to it that these man-hours are spent comfortably and efficiently. Accordingly, after the substance of a paper had been appraised with the help of referees and the paper accepted I had no hesitation in recommending such revisions as would eliminate irritating barbarisms, or would relieve the reader from having to go back and forth over a diffuse discussion in search of a logical sequence, or would clarify an ambiguity, which, however slight, brings a reader to a dead stop. A great fault of a scientist is that he may have so well in mind what he intends to say that he is inattentive to what his sentence actually does say. No editor can be a mind-reader and it is here that his greatest difficulty lies in trying to be helpful.

An editor encounters a perplexing case when a paper contains good data which are very limited in scope and hedged by restrictions on which the author hints that he may publish in subsequent papers. While wondering what to do, an editor may be led to reflect on the state of scientific literature in general. A whimsical address on this was published in the *Journal of Bacteriology* [27, 1 (1934)] under the title "Evolution Toward a Mature Scientific Literature." One who cares to read this address may be amused by the mistake I made in predicting reforms to occur at dates now passed and of which there are no signs after more than a quarter of a century. No one need be reminded of the intolerable jam toward which we are headed. Despite various proposals, in the last analysis the responsibility rests on each author to do his best to make his sector of the scientific literature less like a scrap-heap and more like an edifice.

I seize this opportunity to express my esteem of Rudolph Anderson, the Managing Editor of the *Journal of Biological Chemistry* for almost a quarter of a century. He handled in a gentle, decisive, and efficient way all of his many duties, often with personal sacrifices known only to close associates. It was tragic that near the end of his tenure of office he had to endure a mis-

understanding of his firm stand on restricting the profits of the *Journal* to the use of the *Journal*. Some members of the Society were not aware of the existence of a binding, gentlemen's agreement made at the time when the originally independent *Journal* was put under the Society's care. This agreement, when found, was made known by the old warrior and devoted editor, Vickery. It clarified the atmosphere. Despite the temporary confusion, Rudolph Anderson always commanded the respect of everyone. He is remembered with deep affection by all who knew him well.

A FEW EXPERIENCES IN TWO WORLD WARS

It may be of interest to recall that during World War I the wings, fuselage, and engine-bed of an airplane were made of plywood glued with waterproof casein glue. Makers of this glue imported casein from Argentina because they claimed that domestic batches were not uniform. It fell to the Dairy Division to show that uniform casein can be produced on a commercial as well as a laboratory scale. In the first trial with a huge vat of skim milk I was naively concerned with only one thought; that the isoelectric point of casein must be reached. Therefore, after all the casein appeared to have precipitated, I stubbornly insisted on further additions of sulfuric acid, and much was required to reach the proper pH number of the whey. I did not anticipate, could not explain then, and cannot explain now a critically important consequence. The granular casein, collected on a drain cloth, did not mat together under its own great weight and could be washed practically free of extraneous materials. What we called "grain-curd casein" promptly went into extensive production.

As a preface to the following comments it may be well to remind younger scientists of two organizations in Washington that dealt with scientific affairs during World War II. By Act of Congress (1863), the National Academy of Sciences (hereafter called the Academy) is charged with the duty of investigating and reporting on any scientific, or technological, subject when asked to do so by any Department of the Government. The National Research Council (hereafter called the Council) is an agency of the Academy, which was created during and after World War I to make available all the scientists of the Nation. The Department making a request pays the expenses of the investigation, but the Academy receives no other compensation whatever.

In the peaceful years after World War I, the armed services did not use the Academy and its Council to the extent that they might have. The Academy does not have funds adequate for expensive researches. These were doubtless among the reasons which led some members of the Academy to urge President Roosevelt to establish, within the Government, agencies with power to initiate, and funds adequate to support, research on matters of critical importance in the emergency. In the final organization, the National Defense Research Committee (NDRC) and the Committee on Medical Re-

search (CMR) were coordinate branches of the Office of Scientific Research and Development (OSRD).

Dr. Frank Jewett was the war-time President of the Academy. As a member of the Executive Committee, and in other capacities, I saw him in action and acquired great admiration of his wisdom. His tact was enlivened by a delightful sense of humor that saved many a delicate situation. Dr. Ross Harrison was the calm and judicious Chairman of the Council. I saw little of Dr. Vannevar Bush, Director of OSRD, but saw much of the results of his inspiring leadership and of that of Dr. James Conant, Chairman of NDRC.

Dr. A. N. Richards was Chairman of CMR. Dr. Richards is an extremely conscientious man. He found it difficult to delegate administrative duties and he carried on his own shoulders the planning of several major projects. Among these was the plan to put penicillin into large-scale production. Only a man of vision and tenacity could have fought down the initial, serious doubts of the feasibility.

Dr. Lewis Weed was a member of CMR and also Chairman of the Council's Division of Medicine. This old friend was very helpful to me. He and Richards took me into their confidences so that we might develop ways by which the Council's Division of Chemistry, of which I was Chairman, could be of service.

When I took over as Chairman of the Council's Division of Chemistry of the Academy—National Research Council there was nothing of importance to defense on the agenda of this august institution, which had been established to serve the Government. NDRC had initiated many chemical projects. Later NDRC assigned to the Division some studies that it did not care to support financially and for which the Division procured the services of volunteers. Space is inadequate for comments on these studies or for comment on the work of several committees of NDRC with which I was acquainted.

Malaria was declared to be the most important medical problem. This is suggested, but inadequately, by what happened at Port Moresby. At one time the troops were so incapacitated by malaria that they might have been overcome by the Japanese had not the Japanese been stopped by malaria, dysentery, and consequent over-extension of the line of supply.

There were so many aspects of research on malaria that I must confine attention to only a few of the problems with which chemists were involved. Quinacrine (Atebrin) became the principal antimalarial agent used by our troops. At the beginning, too little was known and when toxic symptoms appeared in the use of domestic samples there arose the suspicion that the toxicity might be because of impurities. The Division of Chemistry was asked to investigate. A critically important item was the preparation of standard samples with which domestic and foreign samples of quinacrine could be compared. In bringing one of these reference samples to a very high

degree of homogeneity, Dr. Lyman Craig became interested in methods and he soon devised his famous counter-current partitioning apparatus. In pharmacological studies under the direction of Dr. E. K. Marshall, Jr. and in clinical studies under the direction of Dr. James Shannon, no substantial differences between the standard, domestic, and foreign samples were found. Obviously quinacrine itself is somewhat toxic.

I am not familiar with contributions by officers in the field to an improved regimen in the use of quinacrine. Their major problem was the enforcement of "Atebrin discipline." At home a great deal of work was organized by the Board for the Coordination of Malarial Studies with Dr. Marshall and Dr. Shannon acting as chairmen of the Panels on Pharmacology and Clinical Studies, respectively. Both of these leaders had the point of view of the chemist and insisted that as much as possible be learned promptly about the absorption, distribution, degradation, and excretion of any compound administered to animals or men. This led to sufficient knowledge to provide an improved regimen in the use of quinacrine.

Meanwhile, searches for a new agent were underway. At the request of CMR, the Division of Chemistry organized the central office of information known as the Survey of Antimalarial Drugs. We were fortunate in obtaining the services of Dr. Frederick Wiselogle to collate the information flowing into this center, a task which he and his large staff performed brilliantly, and in having Dr. Kenneth Blanchard appointed as a free lance to probe wherever his remarkably wide knowledge might lead. Blanchard became invaluable in this capacity. In dealing with commercial firms, whence came a large proportion of the compounds being screened for antimalarial action by use of the avian malaras, the policy was outlined by Dr. Richards. The article of faith was that everyone desired to aid our armed forces by finding, as rapidly as possible, an improved antimalarial agent, and that in the course of the effort there could be maintained, as usual, respect for intellectual and property rights. As developed by consultations between the Chairman of the Division of Chemistry and heads of research divisions of commercial firms, and approved by Dr. Richards, the essentials of the more detailed policy were as follows: Information submitted to the Survey without restriction was circulated by the Survey with proper regard for national security. When information was submitted "in confidence" (as distinct from the Government's "confidential") the restriction was honored, with the understanding that the information must go to those actually working with the compound, and with the further understanding that if a wider circulation were to be deemed advisable a specific request for this would be made. That the policy worked well is indicated by two facts. When the time came for wider circulation, the Survey already had the *carte blanche* of several firms; only one firm held out for a short time and then made the permission unanimous so that when Wiselogle edited the *Tables* for publication he could say "Nothing of any importance has been omitted." When we thanked contributors at the end of the war we received many letters expressing confidence

in the integrity of the Survey and pleasure in having cooperated. The *Tables* contain information on more than 15,000 substances, not all of them definite compounds. For example, it was more economical to demonstrate with a few ducks that Nile mud had no promise than it would have been to engage in lengthy correspondence with its advocate. By the end of the war several compounds had been well documented as being superior to quinacrine (Atebrin). I am not familiar with advances since the war.

The Division of Chemistry was also charged with the organization of additional synthetic work. This began on a voluntary basis and here I made a bad mistake. I assumed that the chemists would have ideas of their own and had best be left alone to develop them. As a matter of fact, the searches for a new antimalarial were, with few exceptions, grossly empirical. It was not until the chemists, pharmacologists, and clinicians of the Board's Panel of Review met with Wiselogle and Blanchard to mull over information in the Survey that there appeared, from time to time, hints that it might be well to expand a particular series of compounds. This required active direction of the synthetic work, which was demanded by the Panel. I was fortunate in persuading Dr. Carl Marvel to take over, and when some matters of administration were straightened out he was appointed. He vigorously organized the synthetic work, the more important parts of which were reviewed by Dr. Robert Elderfield in *Chem. Eng. News* 24, No. 19, p. 2598 (1946).

The following note is too brief to enlighten the historian and too vague for the statesman but it may amuse one or the other.

In the evolution of the malaria program, numerous committees of the Council and individual members thereof organized various projects at the behest of CMR, and finally coordination was achieved by the Board for the Coordination of Malarial Studies. On this Board were representatives of the armed services, the Public Health Service, CMR, and the Council, with Dr. Robert Loeb the vigorous Chairman. By consent of the institutions represented, the Board was an independent board. Funds for meetings and travel, for research and overhead continued to be supplied by OSRD on the recommendation of CMR.

The scientists who operated in this potpourri, while intent on getting things done, were usually and delightfully casual in their indifference to the formalities of administration—until observance of the formalities caused delay. Their reactions were then so typical that there is no need to cite specific examples. The more or less informal and, on the whole, effective cooperation between the various institutions was all to the good up to a point. A decisive point was reached with full appreciation of the fact that a very large proportion of the investigations had come under Government contracts and of the fact that although institutions not under Government control had proprietary interests in certain compounds the values of these compounds as anti-malarial agents were being established at great expense by the Government. There is no need to spell out the possible conflicts of interest or the possibility

that the handling of the situation might set a precedent with political repercussions. The great responsibility for handling the situation wisely led to such a reorganization of the staff of CMR as would bring the details of operations more directly and firmly under the control of CMR.

I was honored by being invited to carry on in the malaria program as a member of the reorganized staff. I declined for two reasons. First: It might be embarrassing to those immediately concerned if a commercial firm were approached by a Government agent in the person of one who had been careful to have it understood that he represented the Council or the Board. One of several instances will suggest this. When officers of a certain firm assumed that a Government agent was proposing expensive work without a proposal for a contract some resentment could be detected in the remark: "The Government can't make us do this." When it was made clear that a request by an advisory committee of the Council was being transmitted there was instant agreement to undertake the work. Be it noted that it would have been inconsistent with "the article of faith" cited in the foregoing account of the Survey, and with the attitude of the Academy, had the transmitter of this request attempted to ferret out the motive of the ready acceptance of the request, or the cost of the work. It was essential to keep negotiations strictly on the scientific basis and in an atmosphere of goodwill, which there is every reason to believe was mutual. Secondly: It seemed inappropriate that an officer of the Academy's Council be involved, if only in a minor capacity, in the day-to-day operation of contracts over which the Academy had no control. President Jewett noted this and, having persuaded me to remain as Chairman of the Division of Chemistry, he thought it unwise for an officer of the Council to risk making a mistake which might appear to involve the Academy in a conflict of interest. I am sure that Dr. Bush and Dr. Richards were sympathetic with this stand. If either one thought it to be stretching an attitude too far, he was kind in not telling me so.

Had another person written the latter part of the preceding note I might be inclined to say that he took himself too seriously and was presumptuous—presumptuous because, so far as I know, no fears materialized under the policy conducted wisely by Dr. Bush and Dr. Richards. However, I risk being accused of foolishness because the incident expresses my strong belief that the Academy will do well to maintain its prestige strictly within the domain of science and technology and to avoid becoming involved, deliberately or by accident, in matters outside this domain.

Of the aviators in the Battle of Britain, Winston Churchill said: "Never in the field of human conflict was so much owed by so many to so few." Results of the great stresses to which these heroic men had subjected themselves worried medical men. Although at that time the functions of the adrenal hormones were not fully understood, the hope was expressed that an adequate supply of the more critically important hormones would relieve the results of stresses to be encountered in the future. This and information that the Germans were collecting adrenal glands from all available sources were

doubtless the stimuli of attempts to provide an adequate supply of the hormones by synthesis. In conferences organized by the Division of Chemistry at the request and with the support of CMR, competent persons from academic and commercial laboratories generously exchanged information while earnestly attempting to accomplish the very difficult syntheses. I am incompetent to say whether or not this exchange of information accelerated the accomplishments attained after the war but it was an outstanding case of cooperation.

Fascinating were the problems considered by the Academy's Committee on Quartermaster Corps Problems and the drive of General Doriot who was in charge of research. Doriot, who had been with Harvard's School of Business, seemed obsessed with the idea that every item which the Quartermaster supplied could be improved. He constantly prodded the committee, of which Lawrence Bass was Chairman, to find those in academic and commercial laboratories who could contribute. Space is adequate for only a story.

Because of his well known ingenuity and competence in the field involved, Dr. Herman Pfund was selected to investigate a highly classified problem. For this, a contract with Johns Hopkins University provided \$10,000. Very soon I visited Pfund to see how he was getting on and, learning that the problem had been solved, I happened to ask the cost of the work. Pfund said that he had picked up needed extras at the Five-and-Ten and had spent 75 cents. This was typical of Pfund. Although out of place here, a pre-war story of Pfund may be told. A student, given the summer job of measuring the temperatures of hot springs, had decided to use a thermocouple. He consulted Pfund on how ice for the reference temperature could be toted all over the wilderness. Pfund told him that he needed only a body that maintains a temperature constant well within a couple of degrees and that a good one could be toted all over the wilderness with an ass.

Early in the emergency the Academy brought to the attention of the Secretary of War the question: What would we do if an enemy were to resort to biological warfare or to use biological agents in sabotage? At the request of the Secretary, an Academy committee, under the chairmanship of Dr. E. B. Fred, considered and reported on this question. There ensued a complicated Government organization. Mr. George Merck was the Director of the War Research Service. Elaborate plans and staffs were at Fort Detrick and elsewhere. The Secretary of War then requested another Academy committee and since Fred had joined Merck's organization President Jewett asked me to be chairman. Very reluctantly, I consented.

There is no sharp distinction between "chemical" and "biological" warfare, but, if we use the latter term as it is commonly understood, we must recognize that among our own people and those of the world there is a greater repugnance for "biological" warfare. One can find some grim humor in the preference for high explosives. However, a nation committed to seeking peace, which includes understanding points of view in other cultures, cannot

afford to play up such grim humor in justifying what may seem logical. Also, as President Jewett remarked, we could not afford to destroy an enemy's crops or cattle; at the end of hostilities it would cost us too much to feed the people. For these and other reasons our Government had no intention to use biological warfare except in a last extremity and the latter intention will be retained as a threat. To implement it the armed services are obligated to be prepared for any plausible eventuality.

In the Academy's committees there was not complete agreement on the feasibility of an enemy's use of certain offensive agents or on the efficacy of certain defensive measures. I suspect that these differences of opinion were not always well weighed by enthusiasts in the Government. Some fantastically elaborate, experimental plants were built without the Committee having been asked for advice. Of course this was the Government's prerogative, but when members of the Committee made constructive suggestions regarding details one sometimes detected the natural, human inclination to interpret such a suggestion as indirect approval of the main objective. Also, some articles published since the war, apparently with the permission of the Government, do not include qualifications which some members of the Committee would have insisted upon had the opportunity to offer them been provided.

The work of the second Committee was made difficult by the scatter of information among several centers of the Government's organization. An especially embarrassing case occurred when the Committee was asked to judge promptly the efficacy of a defensive measure which Intelligence indicated might be necessary. Luck led to the quick assembly of an *ad hoc* committee of persons eminently qualified to give a sound judgment. After great difficulty in assembling the required information they gave a judgment which undoubtedly played a part in saving the Army from what might have been both an unnecessary and an embarrassing action.

This case brought forcefully to my attention the danger of playing with luck in my ignorance and medical matters and I urged President Jewett to appoint another chairman. He appointed the elder medical statesman Dr. Perry Pepper.

To re-enforce what I have said above, with considerable pride and for another reason, I quote from President Jewett's letter discharging me. "Harrison and I appreciate what you have done in handling a most difficult assignment under most trying circumstances. It couldn't have been done better." The last remark was far too generous because one with a job to do should find a new way of doing it if an old way doesn't work well. Lewis Weed had the genius to do just that, as was illustrated many times during the war. He never hesitated to reorganize committees in adjusting to changing conditions and if any rift in the mutual understanding between an advisory committee and a Government agency occurred, he was never timid in making repairs. The way to this was eased by having on committees of the Council's Division of Medicine representatives of the Surgeons General. Such liaison is essen-

tial. President Eisenhower's Executive Order of 10 May, 1956, provides the mechanism for such liaison; its operation depends on the goodwill of all concerned.

I would add this: Although it should be clear that the Academy is concerned with scientific matters only, it would seem wise not to limit this concern too strictly. I may have misunderstood the remarks of an Army officer, but I gained the impression that he regarded scientists to be denizens of the laboratory and considered experiments on the home field to fall within the category of trial tactics which should be the concern of officers only. One could wonder who would advise on the numerous scientific problems arising under field conditions.

I was acquainted with only a limited number of the efforts to aid our armed forces by the results of scientific research. Of these efforts, three general impressions remain vivid:

1. In many a case scientific capital was soon exhausted and raw empiricism became the rule. But with dogged persistence brilliant scientists used their intuitions in designing exploratory experiments. Often these experiments revealed relations of general scientific interest, but too often the practical objective was reached after the war, if at all. In some cases the immediate practical results were impressive. The more outstanding, practical results may have led Congress and the Public to overemphasize research on specific problems. This impression seems to be confirmed by the delay in reaching the present, broader perspective in which basic research has a more prominent place.

2. I saw an abundance of goodwill as made evident by the fact that everyone, without exception, followed advisory committees' requests which I had the honor to transmit in the name of the National Research Council.

3. At the end of the war, in recommending the discontinuance of the Board for the Coordination of Malarial Studies, the members expressed the widely held conviction that, although a committee may serve in an emergency to focus attention on an immediate need, nature has provided no corporate mind.

BIOLOGICAL OXIDATIONS^{1,2,3}

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Because of the problems of limited space and an increasing quantity of papers in the field of biological oxidation, this article is intended only to summarize and evaluate critically what the author considers to be the major developments in the metabolism of oxygen during the last several years. Reference to the previous literature is limited to those papers which are pertinent to the present discussion. Readers are referred to previous review articles (1, 2, 3, 4), two symposia (5, 6), and a colloquium (7) for references to the historical background and earlier literature.

OXIDATIVE VERSUS OXYGENATIVE PATHWAY

Role of oxygenases in biological oxidation.—The oxygenases,⁴ which were first described in mushrooms (8) and pseudomonads (9), now appear to enjoy ubiquitous distribution in animals, plants, and microorganisms and to play an important role in the metabolism of not only various aromatic compounds but also a number of aliphatic compounds as well (10). It has been assumed, however, that these oxygenation reactions are primarily concerned, *in vivo*, with transformations of various essential metabolites and detoxification of foreign substances. Thermodynamically speaking, these reactions appear to be a waste of energy, since no trapping device for energy has been found. When the reduced nicotinamide nucleotides are oxidized by way of the flavoprotein-cytochrome system, the synthesis of at least three moles of high energy compounds (ATP) per mole of NADH₂ results. However, hydroxyla-

¹ This review covers the literature which appeared through September 30, 1961 in the journals available to the author. The selection of papers was largely determined by the present research interests of the author. Much exciting and important work in this field may therefore have been omitted from discussion.

² The following abbreviations are used: DOPA (3,4-dihydroxyphenylalanine); FAD (flavin adenine dinucleotide); FADH₂ (flavin adenine dinucleotide, reduced form); NAD (nicotinamide adenine dinucleotide); NADH₂ (nicotinamide adenine dinucleotide, reduced form); NADP (nicotinamide adenine dinucleotide phosphate); NADPH₂ (nicotinamide adenine dinucleotide phosphate, reduced form).

³ The author is indebted to Dr. Robert K. Gholson for aid in the preparation of the manuscript and to Drs. S. Seno, H. Taniuchi, Y. Nishizuka, and Y. Kojima for helpful discussions and for searching the literature.

⁴ The term "oxygenase," in a broad sense, is applied to any enzyme which either adds both atoms of molecular oxygen to a substrate or adds only one atom of oxygen while reducing the other to water. The former may be designated as "true oxygenase" and the latter "hydroxylase" according to recent usage in the literature.

tion reactions utilizing NADPH_2 or NADH_2 do not seem to be coupled with high-energy bond synthesis and the cell would lose three equivalents of ATP. Oxygenases and hydroxylases may play a regulatory role in energy distribution in the cell, because these enzymes compete with the conventional electron transport system for oxygen and for reduced nicotinamide nucleotides (Fig. 1).

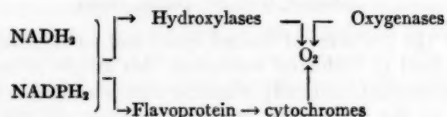


FIG. 1. Competition between oxygenases and the conventional electron transport system.

Oxygenases are not associated with any one specific constituent of the cell, but are distributed in various cellular fractions. For example, homogentisic oxygenase (11) and 3-hydroxyanthranilate oxygenase (12, 13) are both found in the soluble, supernatant fraction of liver cells, whereas kynurenine hydroxylase (14) and the cholesterol side-chain cleavage enzyme (15) are located in the mitochondrial fraction, and nonspecific aromatic hydroxylase (16) is found in the microsomes.

The activity of kynurenine hydroxylase is enhanced three- to tenfold by the addition of 10^{-3} *M* cyanide or azide. This effect may be partly caused by the unique requirement of this enzyme for certain monovalent anions for maximum activity, but may also result from the fact that the cytochrome system is blocked by these inhibitors and, therefore, more reduced nicotinamide nucleotides become available to the hydroxylase.

This phenomenon is of practical importance because, as will be described later, the conventional electron transport system via flavoprotein and cytochromes appears to be much more active than oxygenases under physiological conditions, and therefore the oxygenase activities may be masked unless the cytochrome system is removed or inhibited by some means. During studies on the enzymatic degradation of kynurenic acid, Behrman & Tanaka (17) described an apparent, approximately eightfold stimulation of the primary reaction by the addition of *M*/60 cyanide.

With a similar enzyme preparation from pseudomonads, Hayaishi *et al.* (18) observed that the kynurenic acid hydroxylase activity is located in a soluble fraction of the crude homogenate and that this activity is markedly inhibited in the presence of a particulate fraction. This inhibition could be largely overcome by heating the particulate fraction at 100°C for five minutes. The nature of this inhibition is not clearly understood at the present time, but it seems reasonable to assume that this inhibitory effect is largely due to the presence of a cytochrome system in the particulate fraction which is depleting the system of the reduced form of pyridine nucleotide coenzymes

and oxygen and is therefore competing with the hydroxylases required to initiate kynurenic acid degradation. This supposition is consistent with the observation that the primary reaction of kynurenic acid degradation requires NADH_2 and oxygen (19).

Affinities of oxygenases and oxidases for oxygen and reduced nicotinamide nucleotides.—One of the major factors controlling oxygenase and oxidase activities is their affinity for oxygen and reduced nicotinamide nucleotides. Although a considerable amount of data pertaining to these K_m values has been reported by a number of investigators, there are large discrepancies (see Table I). This is probably due to the fact that the experimental conditions and the methods used by various investigators differ enormously. In general, the K_m values for oxygen obtained with the Warburg manometric technique appear to be much higher than those obtained by polarographic means. This discrepancy may largely be a result of the slow diffusion of gaseous oxygen into the medium which seems to be limiting the rate of the over-all reaction in manometric experiments.

Recent experiments from several laboratories (20, 21, 22) are in reasonable agreement with a value of 2.0 to 0.5×10^{-6} M oxygen for the K_m of cytochrome oxidase. On the other hand, oxygenases such as pyrocatechase and metapyrocatechase exhibit values of 2.3×10^{-5} M and 1×10^{-5} M respectively (23), while the K_m values of tryptophan pyrrolase, 1.4×10^{-4} M (24), and D-amino acid oxidase, 3×10^{-4} M (25), show another tenfold increase.

The K_m for reduced nicotinamide nucleotides of kynurenine hydroxylase is 2.5×10^{-5} M (14), while those of imidazoleacetic acid oxygenase and kynurenic acid hydroxylase are 8×10^{-6} M (26) and 4×10^{-5} M (27) respectively. These figures are comparable to the K_m values for soluble NADH_2 or NADPH_2 cytochrome reductase (Table II). It appears, therefore, that cytochrome oxidase, which is the terminal electron carrier of the conventional electron transport pathway, exhibits a 10 to 100 times higher affinity for oxygen than do the oxygenases, hydroxylases, and other oxidases, while the affinity towards reduced nicotinamide nucleotides is about the same order in these enzymes and the electron transport system.

Quantitative aspects.—Although the significance of various oxygenases in general metabolism has been well established, it is at present difficult to estimate what percentage of the so-called "respired oxygen" is incorporated into cell metabolites and constituents under physiological conditions. Experiments bearing on this problem, using various micro-organisms and plants, have been reported from several laboratories.

When various micro-organisms were grown under different conditions, incorporation of atmospheric oxygen took place to varying degrees. For example, when a *Pseudomonas* sp., a strictly aerobic micro-organism, was grown with aromatic compounds, such as benzoic acid, phenylalanine, or tryptophan as a carbon source, from 4 to 9 per cent of the oxygen of cell constituents was found to be derived from atmospheric oxygen (28). Because CO_2 and water contained much less ^{18}O , most of the incorporation of ^{18}O into

TABLE I
 K_m VALUES FOR OXYGEN OF CYTOCHROME OXIDASE,
 OXYGENASE, AND OTHER OXIDASES

Enzyme	Source	Method	K_m (M)	Reference
Cytochrome oxidase	Pig heart	Polarographic Vibrating, Pt	2.0×10^{-4}	(158)
Cytochrome oxidase	Pig heart	Polarographic Vibrating, Pt	1.0×10^{-4}	(20)
Cytochrome oxidase	Pig heart	Polarographic Rotating, Au	1.86×10^{-3}	(158)
Cytochrome oxidase	Ox heart	Polarographic Rotating, Au	2.39×10^{-3}	(159)
Cytochrome oxidase	Rat liver	Manometric	0.8×10^{-4}	(22)
Cytochrome oxidase	Rat liver	Manometric	5×10^{-4}	(160)
Pyrocatechase	<i>Pseudomonas</i> sp	Polarographic Vibrating, Pt	2.3×10^{-4}	(161)
Pyrocatechase	<i>Pseudomonas</i> sp	Manometric	2.5×10^{-4}	(161)
Pyrocatechase	<i>Pseudomonas</i> sp	Spectrophotometric	3.3×10^{-4}	(161)
Metapyrocatechase	<i>Pseudomonas</i> sp	Polarographic Vibrating, Pt	1.0×10^{-4}	(23)
3-Hydroxyanthranilic acid oxidase	Rat liver Ox liver	Spectrophotometric	1.2×10^{-4}	(13)
Tryptophan pyrrolase	<i>Pseudomonas</i> sp Rat liver	Spectrophotometric	1.5×10^{-4}	(24)
D-Amino acid oxidase	Pig kidney	Manometric	$3.0-1.4 \times 10^{-4}$	(25)

cell material appears to have been a result of direct fixation of atmospheric oxygen. When glucose was used as a carbon source, the extent of oxygen fixation was much less; ^{18}O enrichment was about 0.4 per cent that of the atmospheric oxygen used in the experiment. These observations are consistent with the previous findings that these aromatic compounds are degraded by enzymatic reactions involving fixation of atmospheric oxygen.

Similar observations were made with other aerobic bacteria such as *Serratia plymuthica* and *Mycobacterium phlei*. However, aerobic fungi, including *Neurospora* and three strains of *Saccharomyces*, showed much less oxygen fixation under the experimental conditions employed. Two strains of *Saccharomyces cerevisiae*, namely 59 RT, a wild type, and 59 RA, a so-called "petit" mutant which does not have a cytochrome system, were investigated but both of them incorporated relatively small amounts of atmospheric oxygen, and there was no significant difference between the two strains (10).

On the other hand, when *Escherichia coli*, a facultative aerobic micro-organism, was grown under comparable conditions, ^{18}O enrichment in the cell material was approximately 0.1 per cent or less of that of the atmospheric oxygen. This is almost comparable to the figures which were obtained with Hela cells. When the *Pseudomonas* cells were grown with H_2^{18}O as a medium,

TABLE II
 K_m VALUES FOR NADH_2 AND NADPH_2

Enzyme	Source	K_m (M)		Reference
		NADH_2	NADPH_2	
NADH_2 -cytochrome- <i>c</i> reductase	Pig heart	1.9×10^{-6} (acceptor cytochrome- <i>c</i>) 2.3×10^{-4} (" 2,6-dichlorophenol-indophenol)		(162)
NADH_2 -cytochrome- <i>c</i> reductase	<i>E. coli</i>	5.9×10^{-6}		(163)
NADH_2 -cytochrome- <i>c</i> reductase	Rat skeletal muscle	2.1×10^{-6}		(164)
NADH_2 -cytochrome- <i>c</i> reductase	Calf liver microsome	2.7×10^{-6}		(165)
NADPH_2 -cytochrome- <i>c</i> reductase	Bovine heart muscle		1.9×10^{-4}	(166)
NADH_2 -peroxidase	<i>Streptococcus faecalis</i>	5.9×10^{-6}	5.0×10^{-4}	(167)
NADH_2 -specific diaphorase	<i>Streptococcus faecalis</i>	2.0×10^{-6}		(168)
L-Kynurenine hydroxylase	Rat liver		2.5×10^{-6}	(28)

approximately 54 per cent enrichment was observed. Although these experimental results are still preliminary, it appears that nearly 40 per cent of the oxygen of the cell material could be derived from sources in the medium other than water. However, oxygen tension, pH, the age of cells, and other growth conditions also influence the extent of oxygenase activity. For example, a high concentration of oxygen seemed to favor both growth and oxygen incorporation when *Pseudomonas* was used as a test organism. Leadbetter & Foster (29) examined incorporation of molecular oxygen into cells of *Pseudomonas methanica* and similar soil bacteria utilizing propane and ethane as a principal carbon source, and reported 2.8 to 7.1 per cent of the oxygen of cell material was derived from atmospheric oxygen, while only 0 to 0.32 per cent incorporation was observed when these cells were grown with more oxidized substrates, such as methanol, glucose, etc.

Rittenberg *et al.* (30) investigated the origin of oxygen of cell protein of *E. coli* K-12 employing ^{18}O -labeled water, glucose, and phosphate. Their results indicate that about 65 per cent of the oxygen atoms were derived from water, and 2 per cent from the oxygen of phosphate. The oxygen atoms 1 and 6 of glucose each contribute about 1 per cent to the total oxygen of the proteins and the remaining 30 per cent of the oxygen atoms of the proteins was not accounted for and was presumed to be derived from the other four oxygen atoms of glucose.

The fixation of molecular oxygen into intact cells of plants has been investigated with etiolated corn seedlings and homogenates (31). Chloroform-soluble substances contained a much larger amount of ^{18}O than did the residues from chloroform extraction. It was concluded that some of the oxygen consumed by the seedlings was incorporated directly into organic material through various oxygenases and lipoxidase which were presumed to be responsible for these reactions. It was estimated that lipoxidase may account for 0.4 per cent of the respiratory oxygen absorption of 2.25-day etiolated corn seedlings.

Similar experiments with animal tissues and organs have not been carried out except with Hela cells as cited above. Changes of the proportions of ^{16}O , ^{17}O , and ^{18}O in the respired O_2 and CO_2 have been determined (32).

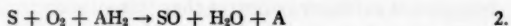
HYDROXYLATION REACTIONS

Mechanism of single hydroxylation.—When Mason and his co-workers (8) described the incorporation of ^{18}O into 3-4 dimethylphenol catalyzed by the phenolase complex, the overall reaction was represented by the following equation.



It soon became evident, however, that this type of reaction always requires an electron donor, and that one oxygen atom is incorporated into

the substrate(s) molecule while the other is reduced to H_2O with the concomitant oxidation of an appropriate electron donor as follows:

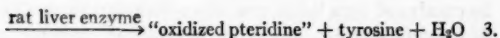


In most hydroxylation reactions, $NADPH_2$ appears to be a specific electron donor, but in some instances, especially in microbial systems, $NADH_2$ is a preferred or specific reducing agent (27, 26). It is interesting to note that the cleavage of the side chain of cholesterol by adrenal cortex extracts requires $NADPH_2$ as a specific electron donor when mitochondria are used, whereas solubilized preparations can use either $NADPH_2$ or $NADH_2$ (33).

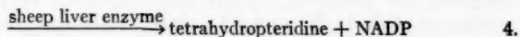
On the contrary, $NADH_2$ appears to function two to six times better than $NADPH_2$ in a crude phenylalanine hydroxylase system prepared from rat, rabbit, and mouse liver (34), whereas in the purified system of Kaufman, involving mixed enzymes from sheep and rat, $NADPH_2$ is much more active than $NADH_2$ (35).

In 1959, Kaufman (35) reported that the direct electron donor in the hydroxylating system of phenylalanine is a tetrahydropteridine and that $NADPH_2$ is acting indirectly, reducing the oxidized pteridine formed in the first reaction.

tetrahydropteridine + phenylalanine + O_2



"oxidized pteridine" + $NADPH_2 + H^+$



Although the natural cofactor isolated from rat liver and synthetic tetrahydropteridines are both active in this system, there are differences in the behavior of the natural and synthetic compounds, and the identity of the natural cofactor remains to be established. The "oxidized pteridine" intermediate in the above scheme was found to accumulate when the hydroxylation reaction was carried out in the absence of phosphate (36). This intermediate is a double bond tautomer of the inactive 7,8-dihydropteridine and is presumably identical with 5,6-dihydropteridine or a compound in equilibrium with the latter.

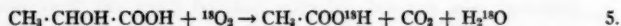
The conversion of DOPamine to norepinephrine involves hydroxylation of the aliphatic side chain rather than the aromatic ring. The enzyme responsible for this reaction was solubilized from bovine adrenal medulla particles by treatment with a detergent and subsequently purified by conventional methods (37). It was found that the electron donor in this aliphatic hydroxylation reaction is L-ascorbate, D-ascorbate, or isoascorbate. Glucoascorbate was also active but other enediols, SH compounds, and reduced nicotinamide nucleotides were inactive. The same enzyme catalyzes the conversion of

β -phenylethylamine to β -phenylethanolamine (38). The activity was reported to be stimulated by ATP and glucose dehydrogenase or by catalase (37), but more recent evidence indicates that this stimulating effect is attributable to a protection of the enzyme from inactivation by H_2O_2 (38). A requirement for fumaric acid has been consistently observed but has not been explained.

The requirement for ascorbic acid as a reductant in the hydroxylating system is not entirely unique since the original experiment by Mason *et al.* (8) with ^{18}O and phenolase was conducted in the presence of a considerable amount of ascorbic acid, although this has not been emphasized in his later review articles.

At one time, ascorbic acid was proposed as a specific cofactor for *p*-hydroxyphenylpyruvic acid oxidase (39). More recent evidence indicates, however, that ascorbic acid is not a cofactor but is protecting the enzyme from inhibition by its substrate (40, 41, 42). Livers of scorbutic guinea pigs contain the same concentration of *p*-hydroxyphenylpyruvic acid oxidase as normal liver but the enzyme in the scorbutic liver is vulnerable to substrate inhibition while that in the normal liver is protected by the ascorbic acid present (43). Dihydroxyfumaric acid serves as an electron donor in the peroxidase-catalyzed nonspecific aromatic hydroxylating system (44).

Although the reactions so far discussed all require an external electron donor, the substrate itself may serve as an internal electron donor for certain reactions. The conversion of L-lactate to acetate plus CO_2 has been shown to be catalyzed by a lactic oxidative decarboxylase which has been purified from *M. phlei* (45, 46, 47). The crystalline enzyme preparation is intensely yellow and contains two moles of riboflavin-5'-phosphate per mole of enzyme. The molecular weight was estimated to be about 260,000. Experiments with $H_2^{18}O$ and $^{18}O_2$ clearly indicated that the mechanism of this unique reaction is as follows (48).

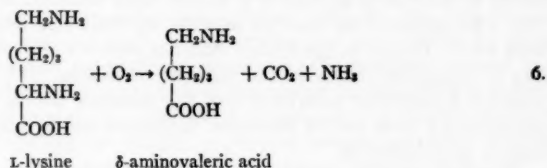


Apparently it has a dual function, i.e., oxidation and decarboxylation of lactate to acetate and CO_2 . A single protein is responsible for both of these activities. Neither pyruvate nor H_2O_2 has been detected as a dissociable intermediate, despite an intensive search for evidence of their presence.

Thus, in the case of lactic oxidative decarboxylase, the substrate, L-lactic acid, accepts one atom of oxygen and simultaneously furnishes two hydrogens to reduce another atom of oxygen by way of the enzyme-bound riboflavin-5'-phosphate. The enzyme-bound reduced riboflavin-5'-phosphate therefore acts as an immediate electron donor, but the substrate furnishes the electrons to be used for the reduction of one atom of oxygen. A similar interpretation may hold for the inositol oxygenase reaction described by Charalampous (49). In this reaction, inositol is oxygenated and dehydrogenated simultaneously, presumably by a single protein.

In the presence of the L-lysine oxygenase of pseudomonads, L-lysine is deaminated, decarboxylated, and oxygenated presumably by a single protein

to form δ -aminovaleric acid. Experiments with H_2^{18}O and ^{18}O were not completely satisfactory because of the interference by nonenzymatic exchange reactions, but available evidence indicates that this enzyme is an oxygenase (50).



Although this latter reaction involves an oxidative decarboxylation similar to that catalyzed by lactic oxidative decarboxylase, the stoichiometry of the reaction does not involve reduction of one atom of oxygen and, therefore, the mechanism could be entirely different from that of lactic oxidative decarboxylase.

In spite of all these increasingly expanding experimental results, no evidence has been reported for the involvement of more than one enzyme in this type of hydroxylating system, or for the occurrence of free intermediates, with the exception of epoxides which have been implicated by several investigators. Bloom & Shull (51) studied the epoxidation of unsaturated steroids by micro-organisms and stated that a microorganism capable of introducing an axial hydroxyl function at Cn of a saturated steroid also effected the introduction of an epoxide grouping "axial" at Cn in the corresponding unsaturated substrate. Taniuchi *et al.* (27) demonstrated that the enzyme, which hydroxylates either 7- or 8-hydroxykynurenic acid to yield 7,8-dihydroxykynurenic acid, appears to produce 7,8-epoxykynurenic acid from kynurenic acid under the same conditions.

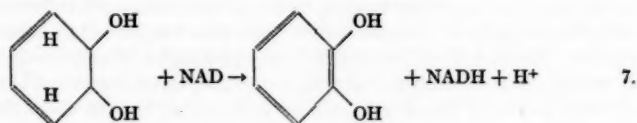
Breuer & Knuppen (52) demonstrated that 16 α ,17 α -epoxyoestratriene-3-ol is produced from oestratetraene-3-ol in the presence of rat liver slices and that the epoxide thus formed is subsequently hydrolyzed to a transglycol. 16,17-epi-oestriol, indicating the presence in animal tissues of a steroid epoxidase and of a hydrolase attacking the epoxide. The chemistry of naturally occurring epoxides has recently been reviewed (53).

Tomkins (54) reports that his steroid hydroxylating system consists of several fractions (presumably protein because of their heat lability) but it is not clear whether these fractions are required for a hydroxylating reaction per se or, as in the case of Kaufman's phenylalanine hydroxylase, are acting indirectly by reducing the ultimate electron donor in the presence of another reducing agent.

The substrate specificity of the microsomal hydroxylating system was further investigated (34). The products were phenols in all cases studied, although dihydrodiols were also produced from naphthalene and quinoline, but the dihydrodiols do not appear to be precursors of the corresponding phenols (55).

Mechanism of double hydroxylation.—It is now well-known that not all hydroxylation reactions are catalyzed by the type of hydroxylases discussed above. The formation of 6-hydroxynicotinic acid from nicotinic acid has been shown to involve hydration of a double bond followed by dehydrogenation (56). The oxygen atom inserted into the nicotinic acid molecule was derived from water. It may be speculated that the presence of a nitrogen atom in the ring of compounds such as the purines, pteridines (57), and nicotine (58) produces a sufficient polarization of the molecule which, together with the presence of a lone pair of electrons, facilitates the hydration of the double bond in such molecules.

Another possible mechanism of hydroxylation is the dehydration of dihydrodiol compounds. Although suggested by some investigators, it has never been demonstrated conclusively by *in vitro* experiments (34). However, enzyme systems which catalyze dehydrogenation of such dihydrodiol compounds have been isolated from mammals and microorganisms (59). Such dehydrogenation reactions yield catechols rather than monophenolic compounds as shown below:



Both *cis*- and *trans*-isomers are dehydrogenated by the same enzyme, although the rate of the reaction with the *trans*-isomer is two- to threefold as fast as that with the *cis*-isomer. Recent investigations from the author's laboratory reveal that in the enzymatic hydroxylation of kynurenic acid to 7,8-dihydroxykynurenic acid, such a mechanism is actually involved (27, 60). The available evidence indicates that kynurenic acid is converted to the 7,8-epoxide in the presence of NADH₂ or NADPH₂ and oxygen; the epoxide is then hydrated enzymatically to form a 7,8-dihydrodiol. The latter compound is dehydrogenated to 7,8-dihydroxykynurenic acid in the presence of a NAD-specific dehydrogenase. Although it is generally accepted that catechol formation involves a single hydroxylation of monophenols, the ubiquitous distribution of the diol-dehydrogenases in nature, together with the recent finding of the 7,8-dihydrodiol of kynurenic acid, makes it attractive to suggest that dihydrodiol compounds may be important precursors in the formation of catechols in nature. The enzymatic conversion of anthranilic acid and benzoic acid to catechol has been studied by several investigators (10, 61, 62). On the basis of the above hypothesis, the role of dihydrodiol as an intermediate in the formation of catechol from benzoic acid and anthranilic acid was investigated. Substrates labeled with ¹⁴C, however, did not yield any labeling

of the carrier, dihydrodiol, when they were converted to catechol in the presence of a cell-free enzyme preparation of a pseudomonad (13).

Other cofactors and metal requirements in the hydroxylation reaction.—Although it is tempting to speculate that enzymatic activation of oxygen is catalyzed by enzymes containing heavy metals such as iron and copper, there is very little clear-cut evidence concerning this point, either by direct analysis or by inhibitor experiments. Kaufman (35), however, reports that phenylalanine hydroxylase of rat liver is stimulated by the addition of Fe^{++} ions after aging or inactivation, the latter brought about by short incubations with versene and cysteine. An inhibitory effect of versene and other chelating agents on the 11β -hydroxylation of steroids has been reported (54, 64, 65).

Certain monovalent anions, such as chloride, bromide, azide, or cyanide, have been shown to be an obligatory requirement for kynurenine hydroxylase activity (14), but this does not seem to be a common property of all the hydroxylases. McCullough (66) and Sloane (67, 68) reported an active cofactor related to *p*-aminobenzoate, which is required for the hydroxylation of aniline and other aromatic compounds, but the exact identification of this compound has not been reported.

Sutton (46) reported the presence of copper in his purified lactic oxidative decarboxylase preparation and Charalampous (49) claims involvement of iron in his inositol oxygenase. However, the role of these metal ions in hydroxylation reactions remains to be elucidated.

The nonspecific aromatic hydroxylase of liver microsomes has been solubilized by Imai & Sato (69) and Krisch & Staudinger (70). The solubilized enzyme which was prepared by incubating microsome preparations with heat-treated snake venom loses almost 80 per cent of its activity upon extraction with a cold acetone-methanol-ether mixture (7:1:2), but almost full activity can be restored by the addition of the lipid emulsion. This observation, together with a similar finding with the unsolubilized preparation (71), suggests an involvement of a lipid-like factor in the microsomal hydroxylation, although stimulation by lipid may be attributable to indirect effects upon the structural integrity of the hydroxylating system, electron transport, or the solubility of nonpolar substrates.

The activity of rat liver microsomal hydroxylase in the hydroxylation of the carcinogen 2-acetylaminofluorene was reported to be stimulated nine- to tenfold by *in vivo* treatment with another carcinogen 3-methylcholanthrene. Available evidence indicates that this increased activity is the result of the synthesis of new enzyme protein (72).

Aliphatic hydroxylations.—The hydroxylation reactions of palmitic acid provide an interesting example of two types of hydroxylation mechanisms. Palmityl coenzyme A can be converted to the β -hydroxy derivative by enzymatic dehydrogenation and hydration, which reactions initiate the well-known β -oxidation pathway of fatty acid metabolism. A hydroxypalmitic acid is also produced by the attack of a hydroxylase in the middle of the

hydrocarbon chain followed by dehydration to yield the Δ 9,10-olefinic acid (73). The formation of oleic and linoleic acids from stearic and oleic acids, respectively, appears to be catalyzed by similar hydroxylases followed by a dehydration process (74, 75).

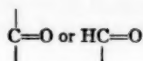
Although circumstantial evidence has recently been accumulating for oxygenative attack on the hydrocarbon molecule (29, 76), direct enzymatic experiments were carried out only recently. Gholson & Coon (77) isolated a cell-free system from a pseudomonad which catalyzed the conversion of octane to octanol, presumably by hydroxylation.

The conversion *in vivo* of aliphatic polyene-carboxylic acids ($H_2C-(CH=CH)_n-COOH$) to the corresponding polyene- ω,ω' -dicarboxylic acids ($HOOC-(CH=CH)_n-COOH$) has been known for more than 20 years (78), but the enzymatic mechanism was investigated only recently. Sorbic acid amide-2- ^{14}C was shown to be converted to muconic acid amide, presumably via ω -hydroxysorbic acid amide (79). Similarly, enzymatic omega oxidation of monocarboxylic acids having 8 to 12 carbon atoms to their corresponding dicarboxylic acids was observed (80). In both cases the primary reaction requires the presence of $NADPH_2$ and oxygen. The latter, which was referred to as omega fatty acid oxygenase, was found in the microsomal fraction of hog, dog, and rat liver, as well as dog kidney.

The hydroxylation of proline to hydroxyproline has continued to be a subject of intensive investigation. Available evidence is consistent with the view that a bound form of proline is hydroxylated, but the exact nature of the reaction is not yet understood (81, 82, 83). Collagen synthesis is augmented by ascorbic acid (84), but it is not clear whether or not this effect is related in any way to the hydroxylation of proline.

Hemoglobin degradation.—The mechanism of oxidative degradation of hemoglobin has been investigated (85, 86, 87, 88). The results of Nakajima indicate that $NADPH_2$, Fe^{++} , and oxygen are required to initiate the primary process. In a model reaction system consisting of hemoglobin, ascorbic acid, and phosphate buffer, one atom of ^{18}O from atmospheric oxygen was shown to be incorporated into the product, biliverdin (89). However, because of the presence of an active exchange between the product and the $H_2^{18}O$ in the medium, these authors suggest the possibility that two or more atoms of oxygen were incorporated into the product.

A major technical problem in this type of work is the elimination or minimizing of nonenzymatic exchange between the oxygen in the organic compounds and H_2O during the incubation and subsequent isolation of the reaction product. This nonenzymatic exchange between oxygen atoms in organic molecules and water is greatly accelerated at acidic or alkaline pH, as was first shown by Cohn & Urey (90) and confirmed by many other investigators. Oxygen atoms in the form of



are especially labile and exchange readily with oxygen atoms of water even at neutral reaction. It is therefore absolutely necessary to carry out isolation procedures at neutral reaction, and at as low a temperature as possible, in order to minimize this complication. Similar difficulties were also reported by Crandall *et al.* (91) and Itada *et al.* (50).

Phenolase.—Kertesz & Zito devised a new method of preparing a highly purified mushroom polyphenol oxidase which appears homogeneous in ultracentrifugation and electrophoretic experiments. By replacing the schlieren bar in the ultracentrifuge with a phase plate, these authors were able to detect the presence of a very light component, which comprised about 1 to 5 per cent of the previously obtained, presumably homogeneous, enzyme preparation (93, 94). In contrast to the previous report, the new preparation, apparently completely free of the light component, has a sharp band at $282\text{ m}\mu$ [$E(1\%) = 26.92$] but the absorption at $250\text{ m}\mu$ is much lower ($E_{280}/E_{250} = 2.31$) and the broad band at about $340\text{ m}\mu$ has practically disappeared ($E_{280}/E_{340} = 69.4$), although at a concentration of 15 mg/ml a solution of the enzyme is still slightly pale yellow. The copper in the polyphenol oxidase seems to be in the cuprous state (94), while that in the blue laccase appears to be in the cupric state and does not form an oxygenated complex (95, 96). On the other hand, Ingraham presented some evidence in favor of the existence of an oxygenated phenolase compound (97, 98).

Perhaps the most confusing and yet important problem in regard to the phenolase complex is the mechanism of its so-called cresolase activity, that is, the introduction of a second hydroxyl group into a monohydroxybenzene derivative in the *ortho* position. According to the enzymatic or direct hypothesis which has been put forward by Dawson, Mason, and others and has recently been reviewed by Mason (1), the enzyme is activated or primed by an *o*-dihydroxy compound or by another reducing agent such as ascorbic acid or hydroquinone. This interpretation is consistent with the presently accepted scheme of hydroxylase reaction mechanism and is supported by experimental evidence with ^{18}O by Mason and his co-workers (8). According to the second hypothesis which was proposed by Kertesz and his associates and which may be called the nonenzymatic or indirect hypothesis, it is the *o*-quinone produced by the oxidation of the *o*-dihydroxyphenol which is the active agent in hydroxylation, and phenolase does not act directly on the monohydroxy compounds (94). It would be interesting to see, as has been pointed out by Kertesz and his associates, whether or not phenolase exists as an oxygenated compound, and to determine the origin of the oxygen incorporated into the hydroxyl group in the absence of a reducing agent.

Karkhanis & Frieden (99, 100) purified mushroom tyrosinase by diethylaminoethyl cellulose column chromatography and found that with this highly purified enzyme preparation the characteristic initial lag with tyrosine was absent. The presence of an inhibitor protein in crude preparations was confirmed by the isolation of an inhibitor protein which is associated with tyrosinase until the final step of purification. Addition of this inhibitor to the

purified enzyme produced an induction period, followed by reduced tyrosinase activity. With DOPA and catechol as the substrate, the inhibitor reduced the rate of the reaction without a lag period, suggesting that the protein inhibitor is causing a lag in the primary monohydroxylation reaction.

OXYGENASES

A new type of catechol cleavage enzyme.—Pyrocatechase was isolated and partially purified from anthranilate-adapted cells of a pseudomonad and was shown to catalyze the oxidative cleavage of the aromatic ring of catechol to *cis,cis*-muconic acid (101). Subsequently the two oxygen atoms incorporated into the product were shown to be derived from atmospheric oxygen through the use of $^{18}\text{O}_2$ and H_2^{18}O (9, 102). Similarly, protocatechuic acid (3,4-dihydroxy benzoic acid) was oxygenated and cleaved between the two hydroxyl groups by protocatechuic acid oxidase from a pseudomonad to form β -carboxymuconic acid (103).

These early observations have led to the general concept that cleavage of the aromatic ring at the C—C bond between the two hydroxy groups represents a major, if not the sole, mechanism by which such catechol derivatives are transformed into aliphatic compounds.

However, during studies on the bacterial oxidation of kynurenic acid, 7,8-dihydroxykynurenic acid was shown to be converted to 5-(γ -carboxy- γ -oxopropenyl)-4,6-dihydroxypicolinic acid (60). Unlike the cleavage catalyzed by pyrocatechase, the fission of the catechol structure in the latter case took place at the C—C bond adjacent to the orthophenol group. These early observations have led us to investigate a similar enzyme which acts upon unsubstituted catechol. The latter enzyme was recently purified from a pseudomonad and was named "metapyrocatechase" (23).

Independently, Dagley and his co-workers have described similar enzymes which act upon catechol and protocatechuic acid. These enzymes were referred to as catechol 2,3-oxygenase and protocatechuic acid 4,5-oxygenase, respectively (104). The results obtained by the British and Japanese investigators suggest that the new metabolic pathway of catechol is as follows (23, 104, 105):

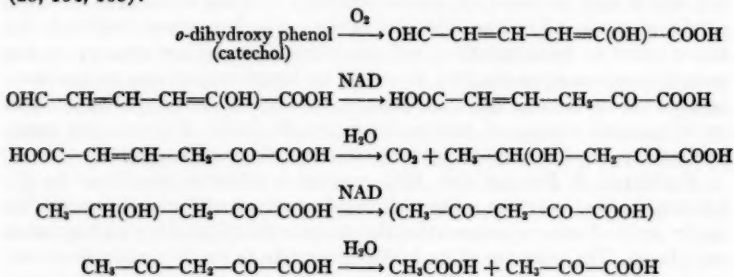


FIG. 2. A suggested new pathway of catechol metabolism.

Metapyrocatechase exhibits properties similar to pyrocatechase with respect to optimum pH (7.5) and K_m values ($0.5 \times 10^{-6} M$) for the substrate. Although both enzymes are inhibited by SH inhibitors, metapyrocatechase is much less sensitive to these compounds. For example, at a concentration of $10^{-6} M$ of *p*-chloromercuribenzoate, CuSO_4 , AgCl_2 , and HgCl_2 , pyrocatechase is inhibited to 77, 69, 100, and 96 per cent respectively, whereas metapyrocatechase is not inhibited by *p*-chloromercuribenzoate or CuSO_4 at this concentration, and is inhibited by AgCl_2 and HgCl_2 to 99.1 per cent and 62.0 per cent, respectively. On the other hand, metapyrocatechase is much more sensitive to oxygen, and inactivation by oxygen can not be avoided by the addition of SH compounds. In fact, the extreme sensitivity of metapyrocatechase to oxygen makes it extremely difficult to purify this enzyme even under nitrogen, while pyrocatechase, which is relatively stable, could be purified 134-fold with an over-all yield of 12.6 per cent. It appears that this extreme instability of metapyrocatechase towards oxygen is the result of some oxygen-sensitive properties other than a sulfhydryl group in the protein molecule.

3-Hydroxyanthranilate oxygenase.—Iaccarino *et al.* (13) described an approximately fortyfold purification of this enzyme from rat and ox liver. This was made possible through eliminating oxygen and by operating in the presence of the substrate Fe^{++} and SH compounds throughout the purification procedure. The purified preparation exhibited absorption maxima at 280 and 400 $m\mu$, but the nature and relation of the latter peak to 3-hydroxyanthranilate oxygenase is as yet unknown. Neither porphyrin nor flavin could be detected in this preparation. The K_m for substrate is calculated to be $7 \times 10^{-6} M$ but the affinity for oxygen is rather low. The K_m value, calculated from the experimental results given, is approximately $1.2 \times 10^{-4} M$ oxygen.

Tryptophan oxygenase (tryptophan pyrrolase).—Further purification of tryptophan oxygenase has been reported from several laboratories (106, 107). Feigleson & Greengard (108) found that the activity of rat liver is confined to the soluble cell sap and can be activated severalfold by the addition of an iron porphyrin activator. Globin acts as a strong inhibitor, presumably through combination with the activator. The same authors showed that the initial phase of the adaptive increase of the tryptophan oxygenase activity can be attributed almost entirely to increased saturation with the cofactor (109). However, the administration of cortisone or reserpine is not accompanied by a significant increase in the relative amount of the activator. In other words, the maximal activity obtained by the intraperitoneal injection of tryptophan or cortisone is approximately the same, but the activity following administration of tryptophan is fully saturated with respect to the cofactor, while the enzyme from the cortisone-treated rats can be activated to the same extent as the enzyme from normal rats by the addition of the hematin cofactor (110). More recently these authors described an approximately 300-fold purification of the apoprotein of tryptophan pyrrolase from

rat liver (111). The purified preparation exhibited essentially absolute heme-tin dependence. It appears that their preparation is the second example of a heme enzyme in which the iron-porphyrin behaves as a dissociating cofactor, since horseradish peroxidase has been for many years the only heme enzyme which could be split by acid-acetone treatment and reconstituted (112). Pitot & Cho (113) described an energy-dependent activation of the same enzyme, which suggests that the activity is released from a particulate bound to a soluble form in the presence of ATP or an ATP-generating system. Fugii *et al.* (114) purified the cofactor and identified it as protoporphyrin IX. Tashiro *et al.* (115) postulated the presence of a similar enzyme in a pseudomonad which acts specifically on D-tryptophan, but isolation of the enzyme and identification of the product(s) have not yet been completed.

CYTOCHROME OXIDASE

The mechanism by which oxygen molecules are reduced by the terminal electron transport system, i.e., cytochrome oxidase, is not yet clearly understood. Since the original discovery of cytochrome-*a₃* by Keilin & Hartree in 1939, it has been generally accepted that the terminal oxygen reductase system consists of cytochrome-*a* which does not react with O₂, CO, or CN, together with cytochrome-*a₃* which reacts with these compounds. However, component *a₃* has never been separated from *a* despite the intensive efforts of a number of investigators [see an excellent review by Lemberg (116)]. Okunuki *et al.* observed that cytochrome-*a* can also react with CO or oxygen when it is reduced to a ferrous form, and that cytochrome-*a* becomes autooxidizable in combination with cytochrome-*c* (117, 118, 119). Furthermore, the oxidation of the reduced form of a partially purified preparation of cytochrome-*c₁* requires the presence of cytochrome-*a* in addition to the component *c* and the rate of the reaction is greatest when the ratio of *a* to *c* is close to unity (120). From this and other supporting evidence, Okunuki *et al.* and Wainio *et al.* independently proposed a so-called unitarian theory which assumes the existence of only one cytochrome, namely the *a* component, acting as an oxidase in the presence of the *c* component (119) or copper (121).

On the other hand, Yonetani (122, 123) obtained a highly purified preparation of cytochrome oxidase from beef heart mitochondria and analyzed the effect of various inhibitors on the α and γ absorption bands. Yonetani's results are in essential agreement with the previous reports by Chance (124) and Smith (125), which suggested the presence of two heme components, *a* and *a₃*, in the oxidase preparation.

A water-soluble cytochrome oxidase has been extracted from cells of *Pseudomonas aeruginosa* and was purified approximately 160-fold by rivanol treatment, ion-exchange chromatography, and zone electrophoresis (126). This purified preparation, which was assessed to be about 70 per cent pure, possessed two kinds of heme in one molecule, one an a_2 -type heme and the other an unknown heme similar to heme *c*.

It has been generally accepted that cytochrome oxidase preparations

contain copper in an amount equivalent to cytochrome-*a*. Sands & Beinert, using an electron spin resonance spectrometer, observed that the copper ion undergoes a valency change during the oxidation reduction process (127). Copper is reduced enzymatically in the presence of cytochrome-*c* and the reoxidation of reduced copper is inhibited by cyanide (128, 129). Although Vander Wende & Wainio (130) claim that the copper in cytochrome oxidase preparations is dialyzable against various chelating agents and the enzymatic activity parallels the remaining copper content, it is generally difficult to dialyze the copper present in cytochrome oxidase preparations (131, 132, 133). Yonetani claims that more than 60 per cent of the copper present can be trapped by bathocuproine sulfonate, a specific cuprous chelating agent, without affecting the velocity of the cytochrome oxidase reaction (134), although Griffiths & Wharton (133) were not able to confirm Yonetani's observation. While the role of copper in the terminal electron transport system has not yet been elucidated, it appears that copper is not directly reacting with oxygen since the oxidase-CO complex is photodissociable but all the metalloporphyrin-CO complexes, with the exception of those containing iron, are resistant to photodissociation.

It has been suggested by a number of investigators that the initial step in the reduction of oxygen by cytochrome oxidase may be oxygenation, binding of O_2 as in the formation of oxyhemoglobin, followed by a rapid internal rearrangement. In order to explain the mechanism of this unique four-electron transfer, Wang & Bringar (135) have synthesized several model compounds consisting of heme, a polycation, and bidentate ligands. The model compounds, which contained 4,4'-dipyridyl or 1,2-di-(4-pyridyl)-ethylene as the connecting ligand, were found to catalyze the oxidation of reduced cytochrome-*c* by air with remarkable efficiency, whereas the polymer containing 1,2-di-(4-pyridyl)-ethane possessed little catalytic activity. These observations show that the conjugated double bond system which links the different heme units together in the active polymers plays an important role in the observed catalysis and provides an interesting model for the four-electron transport system.

METHODS

Rapid progress in the physicochemical methods pertinent to the study of oxygen metabolism has brought about a number of new experimental approaches.

Polarographic methods of oxygen determination.—A suitable method for the measurement of oxygen consumption with the platinum electrode was originally devised by Davies & Brink in 1942 (136). Modifications have been developed by Chance (137) and others, but these methods were subject to various criticisms, despite the fact that they are much more convenient than the conventional manometric technique. The theory and evaluation of various polarographic methods for measuring tissue oxygen tension have been discussed (20, 138). Hagiwara (139) carefully examined and eliminated

sources of error by designing a suitable reaction chamber and by coating the platinum electrode with collodion. A micro-apparatus which permits the measurement of 0.01 mg O₂/hr has been devised (140).

¹⁸O Techniques.—Recent attempts to produce and to utilize oxygen-20 resulted in a disappointing outcome. Oxygen-20, which was expected to be more stable (141), was formed by bombardment of oxygen-18 with tritium and has now been found to have a half life of 13 seconds (142). Through the effort of Israelian investigators (143), a high concentration of H₂¹⁸O is now commercially available, and has been widely used as a tracer of oxygen atoms. More recently oxygen-17 has become available. The latter isotope has the added advantage of having a nuclear spin and can be employed for nuclear magnetic resonance studies.

A comprehensive bibliography of all the papers involving the use of oxygen-18 and -17 has been published (144) with a supplement covering more recent additions through the end of 1960 (145).

The preparation of ¹⁸O-labeled compounds and the methods of analyses of stable oxygen isotopes were reviewed by Samuel (146). Although some attempts were made to make use of gas chromatographic techniques (147, 148), infrared spectroscopy (149, 150), nuclear magnetic resonance spectroscopy (151, 152), and activation analysis (153), the mass-spectrometer is still the most universal instrument for ¹⁸O-analysis. These newer methods of analysis may turn out to be valuable tools in the future, but they need further research and development before being used for tracer work.

Electron spin resonance and nuclear magnetic resonance.—Electron spin resonance and nuclear magnetic resonance spectrometers are becoming valuable tools in elucidating the mechanism of various oxidative processes. Applications of these techniques to biochemical problems are reviewed by Longuet-Higgins (154). Free radical intermediates during peroxidatic oxidations have been detected and identified by means of electron spin resonance spectroscopy (155). Nakamura, using a Varian electron spin resonance spectrometer, describes the oxidative formation of *p*-benzo-semiquinone from *p*-benzohydroquinone in the presence of laccase (156). The kinetic analysis indicates that hydroquinone is oxidized to semiquinone by laccase and the latter dismutates before it is further oxidized to quinone by laccase, the second step being presumed to be a nonenzymatic process. Yamazaki & Piette (157) followed the formation and disappearance of ascorbate free radical, using an electron spin resonance spectrometer adapted with a flow apparatus, and concluded from the kinetic data that a free radical mechanism is the main pathway involved in the ascorbic acid oxidase and peroxidase reactions.

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OXIDATIVE PHOSPHORYLATION^{1,2}

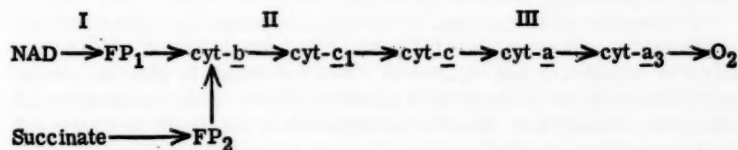
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This review deals with recent research on the mechanism of transformation of respiratory energy into phosphate bond energy; it will also touch on those transport and mechanochemical properties of mitochondria which are associated with the respiratory chain. No attempt will be made to review effects of drugs and endocrine agents, of physiological and pathological states, or of comparative aspects. For recapitulation of the basic advances in the field, the following sequence of reviews and articles provides full historical coverage: Lardy & Elvehjem, 1945 (1); Hunter, 1951 (2); Lehninger, 1951 (3); Lehninger, 1955 (4); Chance & Williams, 1956 (5); Slater, 1958 (6); Lehninger, 1960 (7); and, most recently, the excellent review of Racker (8). For those aspects of the literature relating active transport to molecular patterns of coupled respiration and membrane structure, the reviews of Robertson (9) and Lehninger (10) may be cited.

THE RESPIRATORY CHAIN

Lack of detailed knowledge of the respiratory chain continues to hamper study of oxidative phosphorylation. The sequence and identity of respiratory carriers in mitochondria of higher animals for which appropriate kinetic evidence exists (cf. 5, 11, 12, 13) are given in the following:³



However, this formulation must certainly be revised in the light of recent work, and additional carriers may have to be added. Although some workers have concluded on spectral grounds that cytochrome-*a* and -*a*₃ are separate entities (cf. 5, 11, 14), more recent work on purified preparations of cytochrome oxidase (cf. 15 to 18) suggests there is only a single cytochrome-*a* molecule. An important review by Lemberg on cytochrome-*a* considers this

¹ The survey of literature pertaining to this review was concluded in October 1961.

² Among the abbreviations used in this review are: ATPase (adenosine triphosphatase); DNP (dinitrophenol); NAD (nicotinamide adenine dinucleotide); NADH₂ [nicotinamide adenine dinucleotide (reduced form)].

³ FP₁ and FP₂ represent flavoprotein₁ and flavoprotein₂, respectively.

question in detail (19). Similarly the identity and interactions among cytochromes *c*, *c*₁, and "lipid-cytochrome-*c*" are still uncertain (cf. 12). The interesting experiments of Sekuzu *et al.* (20) and Yonetani (21) on the requirement of cytochrome-*c* for air oxidation of reduced cytochrome-*a* bring into question the sequence of cytochromes shown above.

Also uncertain is the identity of the flavoprotein which oxidizes NADH₂ (cf. 11); several preparations have been isolated which show dissimilar properties (22 to 29). These differ with respect to acceptor specificity and to amytal and antimycin sensitivity and show a puzzling discrepancy as to whether the prosthetic group is flavin mononucleotide (28), flavin-adenine dinucleotide (24, 25, 26), or some other flavin nucleotide (22). The electron carrier function of the nonheme iron of such preparations is still an open question (cf. 11).

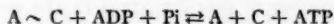
The respiratory chain probably contains additional members not shown above. Ubiquinone (coenzyme Q) is the most prominent candidate; a recent Ciba Symposium considered the role of this and other quinones in respiration (30). Hatefi *et al.* (31, 32, 33) have recently shown that ubiquinone can serve as a carrier to connect the four electron-carrier complexes of the NAD- and succinate-linked respiratory chains to give high rates of nonphosphorylating electron transport. However, kinetic evidence on intact mitochondria and submitochondrial particles (34, 35) appears not to be compatible with the participation of ubiquinone as an obligatory carrier in the respiratory chain, although Redfearn & Pumphrey have shown that its oxidation-reduction state in intact mitochondria does vary with respiratory state in a manner similar to the behavior of accepted carriers (36). It has been suggested that ubiquinone is concerned not in the "main-line" of respiration, but in some corollary function, as in the succinate-linked reduction of NAD (34, 35).

The possible participation of other quinone systems in respiration, such as vitamin K and α -tocopherol, has also been considered (cf. 13, 30). The forms of ubiquinone and vitamin K which are active in phosphorylating respiration may not be the quinone-quinol couples but rather quinone-quinol phosphate couples, with possible participation of phosphate derivatives of the corresponding cyclized chroman forms as intermediates (37 to 42). This view is supported by the isolation of what appears to be a phosphate derivative of vitamin K₁ "naphthotocopherol" by Brodie & Russell from a *Mycobacterium phlei* respiratory system (43).

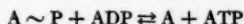
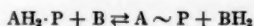
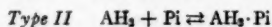
With the uncertainties listed above it is clearly impossible to specify the exact identity of each of the three energy-coupled electron carriers involved in ATP synthesis. Nevertheless there is good evidence that in the NAD-linked chain Site I lies in the span NAD-flavoprotein, Site II in the span cytochrome-*b*-cytochrome-*c*, and Site III in the span cytochrome-*c*-oxygen. The succinate chain contains only the latter two. These sites, which are consistent with thermodynamic considerations, have been experimentally located by direct determination of the P:2*e* ratio of specific spans of the respiratory chain and by determination of the "crossover" points (see 4 to 8).

Reconstruction of segments of the energy-coupled respiratory chain from isolated components is faced by two specters. One is the relatively low specificity of interaction of isolated respiratory carriers; it is possible that two carriers which do not stand in sequence in the intact chain may nevertheless interact with each other *in vitro* and thus lead to an irrelevant *in vitro* reconstruction. The second is the problem of recognizing the chemical devices in the coupled carrier molecules which are responsible for energy conservation. Not a single electron carrier isolated to date has given any recognizable chemical, physical, or kinetic evidence of a potential energy-coupling reaction occurring during its oxido-reduction. They may in fact be degraded molecular forms, possibly equivalent to the chymotrypsin-digested "one-headed" glyceraldehyde-3-phosphate dehydrogenase of Racker and Krinsky (8), which is capable of catalyzing reduction of NAD^+ by free glyceraldehyde but is unable in the presence of glyceraldehyde-3-phosphate to effect the coupled and simultaneous formation of the 3-phosphoglyceroyl-enzyme complex in which the oxido-reduction energy is normally conserved in the native or "double-headed" form of the enzyme. The respiratory carriers isolated to date may contain a natural uncoupling agent, such as a higher fatty acid (cf. 8, 44, 45, 46), which would eliminate a detectable requirement for phosphate or some other component for the oxido-reduction. An alternative possibility is that the coupled respiratory carrier may form an internal or intramolecular high-energy linkage during oxido-reduction which normally may react with an energy-coupling factor, if it is present, but which may be rapidly broken down by water of the medium in its absence.

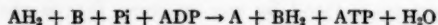
Theories and polymodality of energy coupling.—Most reaction patterns proposed for energy conservation in the respiratory chain are variants of two basic patterns, designated by Slater as Type I and Type II mechanisms (6). In Type I mechanisms it is proposed that some substance (not phosphate), variously designated as "C" [Slater (47)], "X" (also Y and Z) [Lehninger (4, 48)], or "I" [Chance & Williams (5)], combines with the carrier during the coupled oxido-reduction to form a bond between them having a high free energy of hydrolysis, which undergoes phosphorolysis in the presence of Pi and ADP in a secondary reaction to form ATP, as follows:



In Type II mechanisms phosphate is postulated as combining directly with a carrier before or during oxido-reduction, as follows:



The sum of the partial reactions in both cases is



Both types of formulation have precedents in one or another of the "substrate-level" energy-conserving oxido-reductions. For example, the glyceraldehyde-3-phosphate dehydrogenase and α -keto acid dehydrogenase reactions are examples of Type I mechanisms. On the other hand, the intramolecular oxido-reduction catalyzed by enolase is an example of a Type II reaction pattern. Racker (8) gives an important analysis of substrate-level phosphorylations and their relations to respiratory-chain phosphorylation.

The theories proposed by Slater (47), Lehninger (4, 48), Chance & Williams (5), and Boyer (49, 50), among others, are examples of Type I mechanisms, whereas the mechanisms proposed by Lipmann (51), Hunter (2), and the Stockholm group (52, 53, 54), and the quinone mechanisms of Wessels (37) and others (38 to 43, 55, 56) are of the Type II category. However, very recently Ernster has altered his position to include the flavoprotein hypothesis which he and his colleagues have elaborated among the Type I mechanisms (57).

Type I mechanisms appear to be more strongly supported by available evidence. In addition most if not all of the known substrate-level oxidative phosphorylations are of this type. Type II mechanisms often fail to explain how phosphate is first incorporated in "low-energy" form, prior to the oxido-reduction which produces the "high-energy" form. The fact that uncoupled respiration does not require presence of inorganic phosphate (cf. 58) also favors Type I mechanisms.

The differences among various Type I and various Type II hypotheses involve the following critical points: (a) the oxidation-reduction state of the carrier in which the oxido-reduction energy is conserved, (b) the number and sequence of intermediate steps which occur before the end-product ATP appears, and (c) the specific electron carriers which are energy-linked. The oxidized state of the coupled carrier is favored as the "energized" state in the mechanisms of Lehninger (4, 48) and Slater (47); the oxidized state is also the "energized" state of all known substrate-level oxidative phosphorylations for which necessary information is available (cf. 8). On the other hand the idea that the reduced state of the carriers is the "energized" state is favored by Chance & Williams (5) and by Ernster and his colleagues (52, 53, 54, 57). The number of reaction steps in the coupling mechanism will be considered below. In addition to the carriers NAD, cytochrome-*b*, and cytochrome-*a* which have been suggested as probable sites of energy conservation (4, 5, 6, 48), the flavoprotein of the NAD-chain has been favored as a coupling site by Ernster and his colleagues (52, 53, 54, 57) and by Glahn & Nielsen (59). The possible role of vitamin K and ubiquinone as energy-conserving carriers has been mentioned.

The hypothetical mechanisms described above are all examples of "flow-sheet" types of reaction patterns characteristic of classical multi-

enzyme systems, in which there is sequential action of a series of enzymes, each acting on the active site or prosthetic group of the preceding component. However, there are other dimensions to the problem of respiratory energy transformation which are not adequately accounted for in such flow-sheet formulations. Actually two other energy-coupled events linked to electron transport occur in mitochondria in addition to formation of ATP. One of these is the active accumulation of ions, such as K^+ [cf. Robertson (9)], which requires respiration and is uncoupled by agents such as dinitrophenol. The other is the active maintenance of structure of the mitochondrion through respiration-linked mechanical or configurational changes in the membranes which bring about swelling and contraction events [see Lehninger (10)]. Lehninger has recently postulated (10, 60, 61) that energy-coupling in the respiratory chain is trimodal, yielding (a) chemical work in forming ATP, (b) osmotic work, and (c) mechanical work. These three processes are integral, in that high-energy intermediates of respiration are required for each. The mechanisms of mitochondrial ion transport and the membrane structure changes must therefore be provided for in any comprehensive theory of respiratory energy transformation. In terms of this conception, study of ion transport and membrane changes may provide new approaches to the mechanism of oxidative phosphorylation, as in the case of the work on mitochondrial C-factor described below.

Recently Mitchell has proposed a new mechanism for oxidative and photosynthetic phosphorylation, under the designation of a "chemi-osmotic" mechanism (62), which also encompasses the osmotic and mechanochemical changes. It proposes that ATP is formed from ADP and P_i by the reverse action of an "anisotropic" ATPase located in the mitochondrial membrane in such a manner that the OH^- and H^+ removed during the dehydration of ADP and P_i are discharged on opposite sides of the membrane. The ions so discharged are trapped in "sinks" of H^+ and OH^- respectively. It is suggested that these sinks in turn are created by release of H^+ from quinones during electron transport on one side of the membrane and release of OH^- on the other side by reduction of O_2 at cytochrome oxidase. Thus the separation of H^+ and OH^- ions on opposite sides of the mitochondrial membrane which occurs during electron transport, as a result of the specific directional location of the electron carriers, drives the reverse action of an anisotropic, oppositely polarized ATPase in the membrane, which is postulated to be essentially impermeable to H^+ and OH^- . This conception has a number of interesting features. Uncoupling agents such as dinitrophenol are visualized as increasing the permeability of the membrane to H^+ and OH^- , by acting as lipid-soluble proton conductors, thus destroying the ion gradient across the membrane and with it the driving force for synthesis of ATP, without interrupting electron transport. Mitchell has demonstrated that DNP, arsenate, and bis-hydroxycoumarin stimulate proton uptake by intact mitochondria (63), as his theory demands. The chemi-osmotic mechanism also rationalizes the fact that no evidence has yet been found for energy-coupling mechanisms in

isolated respiratory carriers; the carriers would not be expected to contain energy-conserving devices, according to this view. Of great interest also is the fact that the hypothesis can explain the occurrence of selective ion accumulations in the mitochondria, as well as conformational changes in the membranes; the latter are suggested to result from electrical stresses induced by the unequal ion distribution and large membrane potential.

Some possible shortcomings of the chemi-osmotic conception may be mentioned. It fails to account satisfactorily for the more or less exact (maximum) stoichiometry of phosphorylation, particularly for partial systems such as the span $\text{NAD} \rightarrow \text{cytochrome-}c$ and $\text{cytochrome} \rightarrow \text{oxygen}$. The selective sensitivity of the three phosphorylation sites to uncoupling agents (cf. 8) also argues against the hypothesis. Occurrence of oxidative phosphorylation in submitochondrial systems, with badly damaged membranes, would be unexpected according to the chemi-osmotic mechanism. However, despite these and other possible objections, the chemi-osmotic hypothesis demands the closest scrutiny and experimental tests since it, together with the ideas of Lundegårdh (64) and Davies & Ogston (65) (9, 10), provides chemically realistic ways of accounting for the ion distributions which accompany oxidative phosphorylation.

The "partial reactions" of oxidative phosphorylation.—These include the latent ATPase activity, the ATP-Pi^{32} exchange, the ATP-ADP exchange, and the $\text{H}_2\text{O}^{18}\text{-Pi}$ and $\text{H}_2\text{O}^{18}\text{-ATP}$ exchange reactions; all are sensitive to DNP and are presumed to be reflections of the reversibility and multistep nature of the coupling mechanism. Racker has made an instructive comparison of the partial reactions of substrate-level and respiratory chain phosphorylation (8). Presumably these reactions occur at all three coupling sites in the chain, but the contribution from each site to the total observed rate of the partial reactions is rather difficult to evaluate. A second problem is that isotopic exchange reactions must be interpreted with caution since their velocity may depend in a complex way on dissociation constants of intermediate enzyme-substrate complexes, on concentration or availability of cofactors or substrates, and on compulsory sequence of interaction of the components of the system, among other factors (cf. 4, 66, 67).

Submitochondrial systems have proven to be particularly valuable for studies of the partial reactions since they are less subject to interference from extraneous side reactions and permeability or structural effects. The partial reactions have been studied in greatest detail in the digitonin particles of rat liver mitochondria (68 to 78), but important evidence has also come from sonic particles of rat liver (79, 80, 81). It is important, however, to recognize that properties of submitochondrial particles may differ significantly depending on mode of preparation. Differences between sonic and digitonin particles have already been observed (68, 69, 79, 80, 81). As prepared in the laboratory of the reviewers (cf. 69), rat liver digitonin particles oxidize only β -hydroxybutyrate, succinate, and NADH_2 at significant rates, do not show total Krebs cycle activity, show P:O ratios (β -hydroxybutyrate) between

1.5 and 2.0, and are "loosely-coupled." They have undoubtedly suffered some loss of necessary components and may thus be suited for dissociation of reaction steps and for reconstitution experiments. Fugmann & Estabrook (82) have, on the other hand, reported "improvements" in the preparation of digitonin particles to yield material giving P:O ratios approaching 3.0, complete Krebs cycle activity, and "tight-coupling"; in short, these particles behave like intact mitochondria. Variations in preparation methods may account for other differences which have been observed (cf. 78).

ATPase activity.—Recent work, particularly on submitochondrial preparations, provides additional evidence for complexity of mitochondrial ATPase and the probability that this over-all reaction, presumably a reflection of an abnormal or aberrant hydrolysis of some high-energy intermediate in the coupling mechanism, occurs in two or more discrete steps. Adenosine triphosphatase properties have been studied in digitonin particles by Cooper & Lehninger (70), Cooper (74, 75, 76), and Vignais & Vignais (77); in sonic particles by Kielley (80, 81), McMurray, Maley & Lardy (79); and in various preparations by Löw and his colleagues (52, 53). These results have been reviewed by Racker (8).

A new advance has been made by Pullman *et al.* (83), who have separated a soluble ATPase from beef heart mitochondria and purified it to a point approaching homogeneity. The enzyme shows properties very similar to those of particulate systems, with respect to "latency," inhibition by ADP, substrate specificity, stimulation by Mg^{++} and by DNP, and inhibition by azide; but in some other respects, such as the failure of oligomycin and sucrose to inhibit, the enzyme differs significantly from the particulate systems. This soluble ATPase, which is irreversibly inactivated in the cold, is capable of restoring oxidative phosphorylation in heart particles (84) (discussed in the following). The enzyme contains no respiratory carriers and is not stimulated by additions of carriers, nor does it catalyze the ATP- Pi^{32} or ATP-ADP exchanges. An apparently soluble ATPase from rat liver which is stimulated by flavin-adenine dinucleotide has been described by Penniall (85). Phospholipids may also be components of mitochondrial ATPases (86).

Recent work on inhibitors and activators of mitochondrial ATPase activity provides new tools and clues as to mechanisms. The antibiotic oligomycin has been found to inhibit completely both Mg^{++} - and DNP-stimulated ATPase activities of intact mitochondria (87, 88) and of digitonin (60, 89) and sonic (87, 88) particles. It is significant that oligomycin does not inhibit the soluble ATPase of Pullman *et al.* (84), suggesting that the latter enzyme brings about hydrolysis of ATP by breakdown of an intermediate other than that susceptible in particulate preparations. Another new inhibitor of significance is potassium atractylate. Vignais *et al.* (90, 91) have shown that this toxic glycoside from *Atractylis gummifera* (92) inhibits DNP-stimulated ATPase in digitonin particles, but not Mg^{++} -stimulated ATPase; its action on the other partial reactions is similar but not identical to that of oligomycin and is considered further below. Lardy & Connelly (93) have

shown that the antibiotic aurovertin, like oligomycin, inhibits DNP- and Mg^{++} -stimulated ATPase activity and the ATP- P_i^{32} and H_2O^{18} - P_i exchanges. However, oligomycin inhibits ATPase activity elicited by a wide variety of reagents, whereas aurovertin does not inhibit ATPase elicited by aging, by thyroid hormones, by valinomycin or tribromophenol. The two antibiotics are thus presumed to act at different sites; the findings suggest occurrence of at least two enzymatic steps between uptake of P_i and ADP, also proposed by Wadkins & Lehninger (7, 48, 60, 94) and Vignais *et al.* (90, 91).

Sucrose and other polyhydroxylic compounds reversibly inhibit the ATPase of digitonin particles, the ATP- P_i^{32} exchange, and H_2O - P_i^{18} exchange, as well as mitochondrial swelling and contraction (70, 71, 73, 95). Lehninger has postulated that these compounds may act as artificial acceptors in some intermediate group-transfer reaction in the coupling mechanism, but probably not with a phosphorylated intermediate (95).

Aldridge & Threlfall (96) have shown that triethyltin inhibits DNP- and Mg^{++} -stimulated ATPase, as well as the ATP- P_i^{32} exchange and phosphorylating respiration. Its inhibition of respiration is not relieved by DNP, and it is clear that triethyltin is not acting at the same site as oligomycin.

Wadkins has found that arsenate, which uncouples phosphorylation and inhibits the ATP- P_i^{32} exchange, also stimulates hydrolysis of ATP in mitochondria and in digitonin particles (97). Since arsenate stimulation of ATPase was found to be catalytic and other anions did not duplicate its effect, it was proposed that an "arsenolysis" of ATP occurs, in which a high-energy intermediate reacts with arsenate to form an easily decomposed arsenyl derivative. Azzone & Ernster also have made similar observations (98). Arsenate differs in its action from DNP in that it does not relieve inhibition of respiration by oligomycin (99).

There has been some discussion whether Mg^{++} - and DNP-stimulated ATPases are different entities (70, 74, 75, 83); on one hand is the fact that sucrose (70), carrier reduction (see below), atractylate (90, 91), and amyltal inhibit DNP-stimulated ATPase activity but not that stimulated by Mg^{++} ; on the other hand azide, oligomycin (87, 88, 93), and chlorpromazine (52, 53, 100) inhibit both. It is probable that Mg^{++} is necessary in DNP-stimulated ATPase activity regardless of the type of preparation studied (74, 75). The apparently different types of mitochondrial ATPase activity probably share at least one common step, perhaps the azide-sensitive point, but may differ in the step where the aberrant hydrolysis takes place. For example $Carrier \sim I$, $X \sim I$, and $P \sim X$ represent three possible intermediates in mitochondrial ATPase activity, each of which may be capable of undergoing hydrolysis under different conditions. In addition there may be other reactions in equilibrium with these, but not in the main coupling sequence, which could also serve as possible "leaks" (90, 91).

Since some rather striking differences in the sensitivity of the three sites to uncoupling of oxidative phosphorylation have been observed (cf. 8),

comparable differences in properties of ATPase activity at the three sites could accordingly be expected. Myers & Slater (101) found that the stimulation of ATPase of intact rat liver mitochondria by dinitrophenol varies with pH in such a way that three apparently different types of DNP-stimulated ATPase activity could be discerned by means of "difference" curves of DNP-stimulation. Multiple pH optima have also been observed in Mg^{++} -stimulated ATPase activity (101) as well as in net oxidative phosphorylation of ADP (102). From such data Hülsmann & Slater assigned the ATPase activity of pH optimum 6.3 to Site 3, that having an optimum of 7.4, Site 2, and that of pH optimum 8.5, Site 1 (102). However, Chance has questioned these interpretations on kinetic grounds (103, 104).

More recently Hemker & Hülsmann in Slater's laboratory have reported that the earlier conclusions of Myers and Slater drawn from the dinitrophenol data are faulty on the grounds that the apparent differential sensitivity of the three sites to DNP is caused by the differing lipid solubilities of undissociated DNP and its anionic form and the variation of the ratio of these species with pH (105). When due account was made of the concentration of DNP in the lipid phase of mitochondria, in which Hemker & Hülsmann believe the uncoupling to take place, then no essential difference in sensitivity of the three sites to DNP could be found (105). Although the DNP data may now be rejected, other evidence from Slater's laboratory for multiple ATPases, such as the effect of pH on Mg^{++} -stimulated ATPase and on the P:O ratio, is still valid. Lagnada *et al.* (106) have found that brain mitochondria show three distinct pH optima of Mg^{++} -stimulated ATPase, at pH 6.0, 7.2, and 8.4. Iwasa (107) has shown three pH optima in the ATP- Pi^{2-} exchange reaction in mitochondria from *Aspergillus oryzae*. In addition Wadkins, in an entirely independent approach, has shown that arsenolysis of ATP (97) and inhibition of the ATP-ADP exchange reaction by arsenate (108) are characterized by two pH optima. Furthermore Penniall has shown that at a single fixed pH = 8.5, DNP has a polyphasic action, causing release of three distinct ATPase activities at $[DNP] = 10^{-8}$, 10^{-5} , and $10^{-3} M$ (109). These findings must be accepted for the time being as indicating multiple ATPases. Racker favors a single ATPase and suggests that the differential sensitivity of the coupling sites to uncoupling agents may be accounted for if the physical attachment of identical molecules of one ATPase to the three sites differs in strength (8). Whatever the explanation, the pH-activity curves for submitochondrial ATPase (70) and ATP- Pi^{2-} exchange (71), as well as oxidative phosphorylation (68, 110), show high activity over a remarkably broad range of pH.

Cooper (76) has carried out an interesting study of the -SH groups of submitochondrial fragments in relation to the ATPase activity, using amperometric titration with Ag^+ . Titration of approximately one-half of the total -SH groups of the fragments yielded full release of latent ATPase activity, but complexing of the remainder of the -SH groups yielded inhibition of the ATPase. This biphasic role of the -SH groups is strikingly similar

to the findings on myosin ATPase activity (111, 112). They suggest that ATPase activity in mitochondria is normally kept inhibited or latent by the presence of appropriately situated specific -SH groups which "cover" the site where water may interact. It is this class of inhibitory -SH groups which is apparently displaced by dinitrophenol or by low concentrations of Ag^+ , with release of ATPase activity. The second class of SH groups is concerned in the ATPase action per se. Other similarities of mitochondrial and myosin ATPase are described below.

Fluharty & Sanadi (113) showed that arsenite and the arsenite-2,3-dimercaptopropanol complex activate ATPase and uncouple phosphorylation. They propose an interesting mechanism of oxidative phosphorylation involving a vicinal dithiol-cyclic disulfide system and point out the similarities to substrate level oxidative phosphorylations.

The ATP- Pi^{32} exchange reaction.—This reaction has been studied in detail as it occurs in digitonin fragments (71, 77) and in sonic particles (81). In the former, the ATP- Pi^{32} exchange has been found to require the presence of ADP; the rate of the exchange varies with the concentration of ATP, ADP, and phosphate in a complex manner (71). Under certain conditions ADP was found to inhibit the exchange reaction (71), an effect which may be related to the inhibition of ATPase by ADP (70). The exchange is inhibited by DNP, bishydroxycoumarin, pentachlorophenol, gramicidin, *p*-chloromercuribenzoate, azide (cf. 71), oligomycin (87, 88, 93), triethyltin (96), and atractylate (90, 91) and by carrier reduction (see below). Atractylate can inhibit the ATP- Pi^{32} exchange completely in digitonin fragments in concentrations which do not, however, inhibit respiration or uncouple phosphorylation (90, 91); on the other hand, in tightly coupled intact mitochondria, atractylate inhibits the exchange and oxidative phosphorylation. This unique inhibitor may reveal some important relationships.

Little is known about the relative contribution each site makes to the total observed rate of the ATP- Pi^{32} exchange (cf. 77). Löw *et al.* (52, 53) have proposed that Site 1 (NAD-flavoprotein) makes the major if not sole contribution to the exchange since they found that amobarbital inhibited the exchange, whereas antimycin-A did not. However, Wadkins & Lehninger have found that under slightly different conditions amobarbital greatly stimulates the ATP- Pi^{32} exchange reaction whereas antimycin-A inhibits it severely (unpublished data).

Chiga & Plaut (114) described a soluble mitochondrial enzyme, insensitive to DNP, catalyzing ATP- Pi^{32} and ATP-ADP exchange reactions; all three components (ATP, ADP, and Pi) were required for both exchanges. It is not identical with the ATP-ADP exchange enzyme of Wadkins & Lehninger, which does not require phosphate (72). While the enzyme at present does not appear to be involved in oxidative phosphorylation, its remarkable properties should be kept in mind during the course of efforts to reconstitute oxidative phosphorylation.

The H_2O^{18} - Pi and H_2O^{18} -ATP exchange reactions.—Chan *et al.* (73) have

found that the DNP-inhibited O^{18} exchanges occur also in digitonin fragments of rat liver mitochondria and resemble those in intact mitochondria (49, 50, 115, 116, 117), including the fact that the rate of the H_2O^{18} -ATP exchange was much greater than the H_2O^{18} -Pi exchange. Electron transport alone, in the absence of phosphate acceptors, was found to activate the H_2O^{18} -Pi exchange (73); passage of one pair of electrons along the respiratory chain caused the exchange of as many as 30 atoms of O^{18} . The H_2O^{18} -Pi exchange can thus be activated either by ATP in the absence of respiration, or by respiration in the absence of ATP (73). The isolated soluble ATP-ADP exchange enzyme of the digitonin fragments does not catalyze incorporation of O^{18} from H_2O into either inorganic phosphate or ATP (73). It is significant that both oligomycin and aurovertin inhibit the H_2O^{18} -Pi exchange reaction (93), as do azide and sucrose (49, 73). Although it appears certain that the H_2O^{18} -Pi exchange is a part of the ATP-Pi³² exchange, none of the current hypotheses explains the fact that incorporation of water oxygen into ATP proceeds at a much higher rate compared to its incorporation into inorganic phosphate.

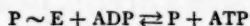
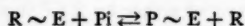
Boyer has shown that the bridge oxygen between the two terminal phosphorus atoms of ATP formed during oxidative (50) or photosynthetic (118) phosphorylation is furnished by the ADP, not by inorganic phosphate. This finding excludes a covalent ADP-enzyme intermediate and is consistent with formation of a covalent phosphorylated intermediate. It is possible, however, that the first covalent bond formed with phosphate might be that between the phosphate and ADP.

The ATP-ADP exchange reaction.—Cooper & Lehninger (71) established that a DNP-inhibited ATP-ADP exchange occurred in digitonin particles and postulated it to be a reflection of an intermediate step in the ATP-Pi³² exchange, which requires ADP as a component. Bronk & Kielley also found a DNP-sensitive ATP-ADP exchange reaction in sonic particles of rat liver mitochondria; it shared pH optima and similar metal ion requirements with the ATPase, the ATP-Pi³² exchange, and oxidative phosphorylation (81). Lehninger & Wadkins have since found that the ATP-ADP exchange reaction occurs in intact mitochondria of all rat tissues tested, including liver, kidney, heart, and brain, as well as in beef heart (108, 119); and in all cases its rate is higher than that of oxidative phosphorylation. In intact mitochondria the exchange is inhibited by both DNP and oligomycin (89, 119) as well as by bishydroxycoumarin, gramicidin, oleate, and arsenate (108). Mitochondria also contain DNP- and oligomycin-insensitive ATP-ADP exchange activity, particularly in the presence of added Mg^{++} (108, 119); presumably the latter type of reaction is caused by enzymes not directly associated with the mechanism of oxidative phosphorylation, such as adenylate kinase (108).

In intact mitochondria (119), and also in digitonin fragments of the type studied by Cooper (78), the presence of inorganic phosphate increases the rate of the ATP-ADP exchange reaction. In the loosely coupled digitonin

preparations of Wadkins & Lehninger (72) or in sonic particles (81) phosphate does not affect the ATP-ADP exchange; these findings parallel sensitivity to oligomycin and indicate that the sites of uptake of P_i and ADP are dissociated in loosely coupled preparations (see below).

Wadkins & Lehninger postulated, on the basis of a detailed study of the exchange in digitonin particles (72), that the terminal steps of energy coupling have the form (48, 72)



a sequence which is in accord with O^{18} data (50). This sequence is supported by the finding that the ATP-ADP exchange can occur in the absence of phosphate (72) and that azide can prevent DNP-sensitivity (72, 108) and by the fact that the enzyme can be separated in a soluble form which does not require phosphate (60, 72). These findings also indicate that the ATP-ADP exchange reaction itself is intrinsically insensitive to DNP, but is normally coupled in close equilibrium with another reaction of the coupling sequence which is DNP-sensitive (60, 72). The findings of Bronk & Kielley are in essential agreement (81).

Very recent work on the exchange has brought out a new relationship: the properties of the ATP-ADP exchange depend on the "tightness" of respiratory coupling in a characteristic and significant manner. In intact tightly coupled mitochondria it is inhibited by maintaining the carriers in the reduced state (120), but in digitonin fragments it is not affected (121). In intact tightly coupled mitochondria the exchange is completely inhibited by oligomycin (89), in digitonin fragments of rat liver mitochondria of an apparently intermediate degree of coupling it is slightly inhibited (78), and in the loosely coupled digitonin particles of Wadkins (89) or in sonic particles (88, 93) it is not affected at all by this antibiotic. The effect of phosphate concentration has been mentioned above.

Kahn & Jagendorf have shown that inorganic phosphate is incorporated into chloroplast grana in a light reaction and combines with ADP in a dark reaction (122). They have isolated an ATP-ADP exchange enzyme from spinach chloroplasts and postulate it to be the terminal enzyme involved in ADP uptake in photosynthetic phosphorylation (123). The similarity in the O^{18} distribution in ATP found in the two processes agrees with this suggestion (50, 118).

Effect of oxidation-reduction state of the carriers on the partial reactions.—Myers & Slater had concluded in 1957 (124) that the respiratory carriers are not molecular participants in the ATPase reaction since the rate of the latter in intact mitochondria was not influenced by oxidation-reduction state of the chain. They suggested that the mechanism of the energy-coupling must include at least one high-energy intermediate between the oxidoreduction reaction and the site of uptake of phosphate, such as the intermediate $X \sim I$ proposed by Chance & Williams on kinetic grounds (5). While their experi-

mental results seemed decisive and have in fact been confirmed under approximately the conditions used (cf. 52, 53, 100, 125), a new complexion was placed on the matter with the finding of Wadkins & Lehninger (121) that the ATPase activity of digitonin particles, in the absence or presence of dinitrophenol, as well as the ATP-Pi³² exchange, is strongly inhibited by maintaining the carriers in the reduced state. Magnesium ion-stimulated ATPase was not affected. The magnitude of the inhibition was found to be dependent on pH and the relative concentration of ATP, phosphate, and ADP. Succinate plus β -hydroxybutyrate as chain reductants produced much larger inhibition than did β -hydroxybutyrate alone. The succinate effect, then puzzling, may now find its explanation (cf. 126) in the succinate-linked reduction of mitochondrial pyridine nucleotides (see following). Wadkins & Lehninger therefore suggested that the respiratory carriers are participants in the ATP-Pi³² exchange and ATPase reactions and that the energized state of the carriers is the oxidized state (121), contrary to the hypothesis of Chance & Williams (5).

The findings of Wadkins & Lehninger have now been confirmed in Slater's laboratory by Chefurka (126), by Vignais & Vignais (77), and by Iwasa (107), with substantially similar findings (see also 127). Relatively high concentrations of cyanide or carefully induced anaerobiosis were essential prerequisites for inhibition. Bronk & Kielley (128), Löw *et al.* (52, 53), and Siekevitz *et al.* (125) have also observed striking effects of oxidation-reduction state of the chain on the ATP-Pi³² exchange and the ATPase activity. The Stockholm group found that quinacrine, promazine and its derivatives, amobarbital, antimycin-A, and cyanide show characteristic effects on these partial reactions. They postulated a mechanism of oxidative phosphorylation involving a phosphate derivative of reduced flavoprotein as an intermediate at Site 1, which they believe is responsible for most if not all of the observed ATPase and ATP-Pi³² exchange activity in mitochondria. In this connection, Penniall has found that flavin-adenine dinucleotide stimulates ATPase of mitochondrial extracts (85).

Reduction of the carriers does not influence the ATP-ADP exchange in digitonin particles (121), but inhibits it severely in intact mitochondria (120).

Although findings in a number of laboratories have now made it clear that the oxidation-reduction state of the carriers does profoundly influence the ATPase and the exchange reactions, thus indicating that the carriers probably participate in one or more of these reactions, precise interpretation of all the reported effects is rather difficult, for reasons which have been summarized by Wadkins & Lehninger (121). Vignais & Vignais have also presented an analysis of the problem (77). Chance & Hollunger (129) have proposed that inhibition of the ATP-Pi³² exchange by reduction of the carriers does not necessarily exclude $X \sim I$ as an intermediate, but may actually constitute evidence for its occurrence, on the ground that availability of free X, presumed to be required for the exchange, may be determined by the oxidation-reduction state of the carriers. While their mechanism could explain

inhibition of the ATP-Pi³² exchange it cannot account for the fact that reduction of the carriers produces simultaneous inhibition of the ATPase activity, the ATP-Pi³² exchange, and the ATP-ADP exchange.

"Tightness" of respiratory coupling.—The inhibition of tightly coupled but not of loosely coupled respiration by oligomycin (87, 88, 93) and atractylate (90, 91) directs interest toward the little-studied problem of the mechanism of loose and tight coupling (5, 13, 130). Fresh intact mitochondria show tight coupling, i.e., respire at very low rates unless phosphate acceptor is present. Addition of a true uncoupling agent such as DNP to mitochondria in the controlled (i.e., ADP-less) state "releases" respiration, presumably by causing breakdown of the same intermediate (Carrier~X or X~I) involved in DNP-stimulated ATPase activity. Such release of respiration may be considered a "partial reaction" of oxidative phosphorylation, with a mechanistic relationship to ATPase activity. On the other hand, mitochondria from some insects (cf. 13, 131) show loose coupling, as do those from hyperthyroid liver (132); they respire at maximal or nearly maximal rates in absence of phosphate acceptor yet yield high P:O ratios when acceptor is added. Sonic and digitonin particles are also loosely coupled.

Remmert & Lehninger have found that a heat-labile nondialyzable fraction extracted from rat liver mitochondria (called R-factor) causes loose coupling when added to rat liver mitochondria or to digitonin particles, without producing substantial uncoupling, an effect not simulated by agents such as DNP (133). They postulated that loose coupling could be brought about by competition between water and ADP for a phosphorylated intermediate, such as E~P. In the absence of ADP, E~P may be hydrolyzed, causing release of respiration, whereas when ADP is present it may compete successfully with water for E~P to form ATP. Van den Bergh & Slater found that a soluble factor which restores phosphorylation to respiring particles from *Azotobacter vinelandii* also increases the acceptor control ratio (134).

Some of the properties of the controlled (or ADP-less) state of respiration do not seem to be completely consistent with traditional views of uncoupling action. Gregg *et al.* (135) have found, for example, that DNP does not release controlled or ADP-less respiration in housefly mitochondria, although it uncouples and also greatly accelerates the ATPase activity. On the other hand, brain mitochondrial ATPase is not usually stimulated by DNP, which does, however, uncouple phosphorylation (cf. 106). Lehninger & Gregg (136) have found that Mg⁺⁺ does not release respiration in digitonin fragments, although Mg⁺⁺ greatly stimulates ATPase activity in these particles; this indicates strongly that the intermediate undergoing hydrolysis in Mg⁺⁺-stimulated ATPase is not an intermediate in respiratory coupling. On the other hand, the intermediate which is sensitive to DNP is apparently a common step in both ATPase and release of respiration. Phosphate and arsenate also cause release of respiration.

The action of oligomycin and atractylate shows tight coupling to be

linked specifically to certain partial reactions. Oligomycin inhibits the tightly coupled respiration of intact mitochondria, as well as the DNP-stimulated ATPase, the ATP-Pi³² exchange, and the ATP-ADP exchange. The respiratory inhibition can be relieved by DNP. On the other hand, oligomycin does not inhibit the loosely coupled respiration of digitonin particles, nor the ATP-ADP exchange in such particles; but in this case it does uncouple phosphorylation and inhibits ATPase and the ATP-Pi³² exchange (87, 88, 89, 93). Atractylate also inhibits the tightly coupled respiration of intact mitochondria as well as the DNP-stimulated ATPase and ATP-Pi³² exchange; the respiratory block can also be relieved by DNP. On the other hand, atractylate does not inhibit either respiration or phosphorylation in loosely coupled digitonin preparations, but unexpectedly it does inhibit the ATP-Pi³² exchange in these circumstances (90, 91). The relationships between tightness of coupling and the action of these inhibitors, as well as aurovertin (93), deserve the closest attention with regard to mechanism.

COUPLING FACTORS, RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION, AND INTERMEDIATES

Some significant successes in dissociating phosphorylating systems of mammalian mitochondria and microorganisms into two or more fractions which can be reconstituted to restore oxidative phosphorylation or its partial reactions, or both, have been reported. Some of the enzymatic capabilities of "coupling factors" have been established; further exploration may lead ultimately to recognition of the chemical mechanism of intermediate reactions in oxidative phosphorylation. However, increases in P:O ratios or reconstitution of partial reactions by coupling factors must be interpreted with caution. Such coupling factors may of course actually be intermediate enzymes directly participating in the coupling mechanism, but it is also possible they may increase P:O ratios by other mechanisms, such as (a) removal of an inhibitor or uncoupling agent, (b) "tightening" of membrane structure to bring together pre-existing but nonreacting enzymes, and (c) inhibition of an uncoupling or interfering reaction.

Factor of Linnane & Titchener (Coupling Factor II).—Linnane & Titchener (137) have prepared a modified form of the "electron transport particle" (ETP_b) (138) which oxidizes NADH₂ and succinate, but no longer is capable of coupling phosphorylation to respiration. A soluble heat-labile fraction recovered from the sonic treatment used to prepare the modified electron transport particle was found to restore phosphorylation in the latter, yielding a P:O ratio of 1.0 for NADH₂ and of 0.6 for succinate. The magnesium ion is a critical factor in the binding of this coupling factor to the particles; ethylenediamine tetraacetic acid was essential to remove it from the particles. The rather unstable factor was purified some eight- to fifteenfold; its assay was difficult since excess concentrations uncoupled phosphorylation. Although some ATPase activity could be observed in some preparations,

this did not appear to correlate with recoupling activity. This factor may be identical with the Coupling Factor II of Penefsky *et al.* (84).

Coupling Factor I (soluble ATPase).—Pullman *et al.* (83) have separated and purified a soluble DNP-stimulated ATPase from extracts of beef heart mitochondria subjected to mechanical disruption. Addition of this enzyme (designated Coupling Factor I) restores phosphorylation in the insoluble heart mitochondrial residue left by the mechanical treatment used to prepare the ATPase (84). The particulate fraction of beef heart mitochondria remaining after removal of the soluble fraction is able to oxidize succinate, β -hydroxybutyrate, isocitrate, and glutamate, with little or no phosphorylation of ADP. However, addition of the soluble ATPase to such particles restored some phosphorylation, with P:O ratios up to 0.8. The coupling factor also reconstituted the ATP-Pi³² exchange reaction. The phosphorylation site restored is apparently Site 2 (i.e. that lying between cytochrome-*b* and -*c*), since oxidation of succinate and of β -hydroxybutyrate yielded about the same P:O ratio; no phosphorylation could be detected with ascorbate and cytochrome-*c*. During purification of the soluble factor, the ATPase activity became more purified than the coupling activity, suggesting loss of an inhibitor of ATPase during the purification. Both activities showed the characteristic cold-lability and both increased on aging at higher temperatures; in most respects the data indicate identity of the ATPase and the coupling factor, which is apparently not identical with the factor of Linnane & Titchener (137).

The soluble ATPase showed no activity in either the ATP-Pi³² or ATP-ADP exchange reactions. Neither the enzyme preparation of Wadkins & Lehninger (72) nor the ATP-ADP-Pi exchange enzyme of Chiga & Plaut (114) was able to replace Coupling Factor I in recoupling phosphorylation in the heart particles. However, very recently it has been found by Racker (139) that the soluble ATPase does show some ATP-ADP exchange when it is incubated with another fraction from beef heart, itself inactive in this reaction, a finding which may provide some element of unification of work on the ATPase and the ATP-ADP exchange enzyme described below.

Racker and his colleagues (83, 84, 139) have obtained a second protein from beef heart mitochondria, named "Coupling Factor II," which is required under certain circumstances, together with Coupling Factor I, in restoration of phosphorylation in suitably treated submitochondrial particles from beef heart. They suggest it is identical with the factor of Linnane & Titchener (137) described above. They have identified a third factor from beef heart mitochondria relevant to the action of the soluble ATPase, namely, a heat-stable inhibitor of the ATPase activity, which "masks" ATPase activity but does not prevent its action as a coupling agent (140).

The soluble ATP-ADP exchange enzyme; "noncombinable" and "combinable" forms.—From digitonin fragments of rat liver mitochondria in which the ATP-ADP exchange reaction is inhibited by DNP, Wadkins & Lehninger (72) extracted, after acetone treatment, a soluble form of the ATP-ADP exchange enzyme in 90 per cent yield. The activity had an absolute depend-

ence on Mg^{++} or Mn^{++} , did not require phosphate or any dialyzable cofactors, and was sensitive to heavy metal reagents. The soluble enzyme showed no sensitivity to dinitrophenol, azide, or oligomycin (60, 89). Using the ATP-ADP exchange activity as a basis for assay, the enzyme was purified nearly 150-fold, approaching homogeneity (60, 141). The purified preparation showed no ATPase, ATP- P_i^{32} exchange activity, adenylate kinase, or protein phosphokinase activity nor a requirement for phosphate; it is obviously not identical with the ATP-ADP- P_i^{32} exchange enzyme of Chiga & Plaut (114).

The partially purified ATP-ADP exchange enzyme was found by Wadkins to increase the P:O ratio of β -hydroxybutyrate oxidation by digitonin particles of rat liver mitochondria which had been exposed to an excess of digitonin (7, 60). In such tests the P:O ratio of the unsupplemented particles was between 0.8 and 1.2; addition of the ATP-ADP exchange enzyme purified up to sixty- to seventyfold gave significant increases of from 0.3 to as high as 1.0. Wadkins & Lehninger (142) showed that the DNP-insensitive soluble exchange enzyme has another property which permitted study of reconstitution: it can be made DNP-sensitive again by adding it to digitonin particles of rat liver mitochondria. This "recombination" effect did not occur in the presence of azide, which normally blocks the inhibitory action of DNP on the exchange in the fragments, nor did it occur on addition to aged, non-phosphorylating particles. These experiments thus demonstrate reconstitution or reattachment of the soluble ATP-ADP exchange enzyme to DNP-sensitive sites in the coupling mechanism. This effect is specific; DNP-sensitivity could not be conferred on soluble adenylate kinase, for example.

Dinitrophenol sensitivity could not, however, be restored to more highly purified samples of ATP-ADP exchange enzyme [\sim 150-fold (60, 142)]. The recombination phenomenon thus occurs only with crude or partially purified preparations; it was suggested that some necessary component for the recombination process was separated or destroyed during further purification (142). This form of the ATP-ADP exchange enzyme is termed the "non-combinable" form.

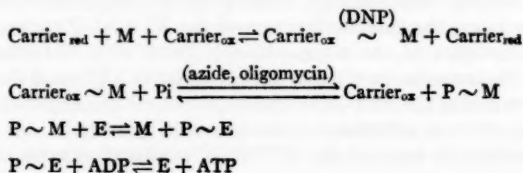
A second soluble form of the ATP-ADP exchange enzyme (termed the "combinable" form) has been separated from mitochondrial extracts by Wadkins & Glaze (60, 143) using a different purification scheme. Such preparations, purified about 300-fold, show properties very similar to the noncombinable ATP-ADP exchange enzyme described above, except in one respect. This form of the enzyme is capable of "recombination" with digitonin fragments to reconstitute its DNP-sensitivity at all stages of purification. It appears possible that the combinable form of the ATP-ADP exchange enzyme may undergo dissociation or degradation to the non-combinable form; the combinable form of the ATP-ADP exchange enzyme may be a complex of the noncombinable form with M-factor (94) (see following). As proved to be the case with the soluble ATPase of Pullman *et al.* (83), purification of the ATP-ADP exchange enzyme often yielded more than the starting activity; mild heating also increased activity.

The combinable form of the ATP-ADP exchange enzyme shows some

properties which are similar to those of the soluble ATPase of Pullman *et al.* (83) in that it can reconstitute oxidative phosphorylation and shows "latency" phenomena. These factors are not identical, however, since the combinable form of the ATP-ADP exchange enzyme shows no ATPase activity and is not cold sensitive. It is possible that Coupling Factor I and the ATP-ADP exchange enzyme may be active in restoring activity of different phosphorylation sites in the respiratory chain. Racker's recent report that ATP-ADP exchange activity can be observed in preparations of the Coupling Factor I when another fraction from beef heart mitochondria is added suggests that the ATP-ADP exchange activity of the soluble ATPase may be "latent." It is thus conceivable that the enzyme causing the ATP-ADP exchange is part of the soluble ATPase. Because of organ and species differences in the source of these enzymes, resolution of the question will require much further work.

M-factor.—Wadkins & Lehninger (94) found that extracts of acetone-dried mitochondria contained a factor which increased the fraction of ATP-ADP exchange activity of digitonin particles which could be inhibited by DNP. This factor, designated M-factor, was purified some sixtyfold. In this form the preparation had little or no ATP-ADP exchange activity itself, failed to catalyze the ATP-Pi²³ exchange, and showed no ATPase activity. The ability of M-factor to increase DNP-sensitivity of the ATP-ADP exchange in the particle suggested the possibility that it is the factor which was lacking in the highly purified noncombinable ATP-ADP exchange enzyme preparations (60, 142) described above. However, efforts to effect restoration of DNP-sensitivity by adding together noncombinable ATP-ADP exchange enzyme, M-factor, and digitonin particles gave erratic and equivocal results. It appears possible that the noncombinable form of the ATP-ADP exchange enzyme may be damaged or degraded so that it still shows exchange activity but is unable to react with the preceding component in the coupling sequence.

Since the soluble ATP-ADP exchange enzyme does not catalyze the ATP-Pi²³ exchange, does not contain respiratory carriers and is not influenced itself by dinitrophenol or by oligomycin, it has been suggested by Wadkins & Lehninger (7, 60, 94) that the coupling mechanism contains at least two protein components, one of which is the exchange enzyme. The other component(s) is suggested to be those moieties which can interact with carrier, with oligomycin, and with dinitrophenol. Since M-factor can increase the DNP-sensitivity of the ATP exchange, it was suggested (7) that M and the exchange enzyme (E) may be related as follows:



C-factor.—Lehninger & Gotterer have found that reduced glutathione (GSH) detaches a heat-labile protein from rat liver mitochondria which is required to bring about contraction of glutathione-swollen mitochondria by ATP (144, 145). This "contraction-factor" (or C-factor) has been extracted from mitochondria and purified 130-fold by Neubert & Lehninger (60, 146). C-factor activity is found in mitochondria of all tissues of mammals so far examined (147). Neubert and Gregg (cf 60) have found that highly purified C-factor preparations increase the P:O ratio of digitonin fragments. C-factor may contain or be identical with one of the coupling factors described above, or may act by "tightening" the structure of the fragments. It may be pointed out that Penefsky *et al.* (84) have reported that when Coupling Factor I is added to beef heart particles to recouple phosphorylation, it also produces aggregation or some other change influencing the optical density.

Possible intermediates.—Suelter *et al.* (148) have shown that respiring mitochondria in the absence of ADP rapidly incorporate P_i^{32} into an insoluble, labile, covalently bound form which could be extracted with urea-Triton mixtures. The amount incorporated was of the same order of magnitude as the cytochromes; kinetics and specific activity were consistent with the view that it is a precursor of ATP. The bound phosphate exchanges rapidly with unlabeled P_i and ATP. Its formation was inhibited by cyanide, amytal, Ca^{++} , and *p*-chloromercuribenzoate, reduced by DNP, but not inhibited by oligomycin. The bound P is converted to P_i at neutral and alkaline pH and by bromine; it is probably not in the form of phosphoserine or phospholipid. It is tentatively suggested that the P^{32} -labeled material is the long sought intermediate of oxidative phosphorylation. Pressman & Dallam have independently described apparently similar rapidly labeled phosphorylated components of mitochondria which could be separated chromatographically (149). Kondrashova also reported evidence for a very labile structure-bound phosphorylated intermediate at the Moscow Congress (150).

Purvis has presented evidence for occurrence of chemical species of NAD and NADP which are not reactive with simple dehydrogenases (151). Although Klingenberg *et al.* could not confirm these observations (cf. 13), the possibility that the pyridine nucleotides may occur in covalently bound but labile forms should not be dismissed because of their potential relevance to the forms $NADH_2 \sim I$ or $NAD \sim I$ which have been postulated as high-energy intermediates.

Reconstitution in microbial systems.—Oxidative phosphorylation has been observed to occur in extracts of many different micro-organisms (cf. 8). Such preparations possess respiratory chains which may be simpler or at least different from those in animal mitochondria and usually give P:O ratios below 1.0, suggesting the possibility that they may contain only one or two phosphorylation sites. Phosphorylation in bacterial extracts is usually insensitive to dinitrophenol, except for preparations from *Alcaligenes faecalis* (152), *Saccharomyces cerevisiae* (153), *Proteus vulgaris* (154), *Mycobacterium phlei* (155), and *Aspergillus oryzae* (107). There is little published informa-

tion on the partial reactions of oxidative phosphorylation in microbial systems, except for reports on *Aspergillus oryzae* (107) and *Micrococcus lysodeikticus* systems (156). Several of the bacterial systems can be fractionated into two components; in most cases both fractions are required to reconstitute respiration as well as phosphorylation (see also 157, 158).

Pinchot has shown that oxidative phosphorylation in a system from *Alcaligenes faecalis* requires at least three components: washed respiratory particles, a soluble heat-labile, nondialyzable component, and a polynucleotide (152, 159, 160, 161). The complete system forms ATP coupled to oxidation of NADH_2 with rather low P:O ratios (152). A unique feature is the requirement for the polynucleotide, which is resistant to ribonuclease and deoxyribonuclease, but which yields adenine and guanine on acid hydrolysis (152). It may be replaced to some extent by poly A, poly U, or poly C synthesized by the action of the polynucleotide phosphorylase from this organism (159, 160, 161). The function of the polynucleotide is to bind the soluble factor to the particles (162). The sensitivity of the system to DNP is apparently related to this binding reaction. Pinchot has recently shown that when aerobic incubation of the particulate complex with NADH_2 is carried out in a medium free of phosphate and ADP, followed by removal of the particles, the soluble fraction alone can then form ATP^{32} when supplemented with Pi^{32} and ADP (162). These findings suggest that a nonphosphorylated high-energy intermediate of oxidative phosphorylation (i.e. equivalent to Carrier~I or X~I) is first generated by respiration in the absence of phosphate and becomes soluble. A soluble terminal kinase then makes possible formation of ATP from Pi and ADP. The latter transfer is not sensitive to DNP.

Brodie has continued work on the role of vitamin K and other naphthoquinones in oxidative phosphorylation in cell-free extracts from *M. phlei*. This system, which earlier work had shown could be resolved into a soluble and a particulate fraction, is inactivated by irradiation at 360 $\text{m}\mu$ (163, 164). Both respiration and phosphorylation are restored by vitamin K and its homologues containing a 2-methyl group and an isoprenoid side chain of at least five carbon atoms at C-3; both are also restored by a naturally occurring naphthoquinone isolated from this organism. Menadione, lapachol, and other homologues without side chains restore only oxidation, not phosphorylation, consistent with the view that formation of the chroman derivative of vitamin K by cyclization of a loop of side chain is essential for restoration of oxidative phosphorylation. Ubiquinone is inactive.

The *Mycobacterium phlei* system incubated anaerobically with malate, and Pi^{32} forms a labile phosphorylated compound of vitamin K, (40, 165). The spectrum of this compound is similar to that of the vitamin K_1 "naphthotocopherol"; on nonenzymatic loss of phosphate the spectrum reverts to that of the quinone form of vitamin K_1 . When the P^{32} -labeled "intermediate" is incubated aerobically with ADP and the extract, labeled ATP is formed. However, its specific activity was found to be only a very small fraction of

that expected, and it cannot yet be concluded that the material is an intermediate in oxidative phosphorylation.

Stadtman has studied the reduction of glycine in extracts of *Clostridium sticklandii* by 1,3-dimercaptopropanol, a reaction which proceeds with formation of one mole of ATP (166, 167). This reaction is inhibited by antimycin-A, by menadione, and by irradiation at 360 m μ , suggesting participation of vitamin K or a quinone. Such extracts have been found to contain a system which hydrolyzes *p*-nitrophenyl phosphate, but no other phosphate esters. This reaction requires the presence of a sulfhydryl compound and of menadione; the latter cannot be replaced by phthiocol, lapachol, or vitamin K₁, but can be replaced by 1-thioesters of menadione. Actually such thioesters are formed in the reaction medium on adding menadione and the required thiol and appear to be the form in which menadione activates the *p*-nitrophenyl phosphatase. The findings suggest that normally a phosphate ester of a natural quinone is formed during glycine reduction as a precursor of ATP, and that the *p*-nitrophenyl-phosphatase activity represents an uncoupling reaction. Stadtman's highly interesting findings deserve more attention in view of Brodie's work on the phosphate derivative of vitamin-K naphthotocopherol.

Ishikawa & Lehninger (156) have found that a particulate fraction from protoplasts of *M. lysodeikticus* can respire but is unable to phosphorylate unless a soluble, heat-labile, nondialyzable factor is added; the latter does not affect respiration. The complete system yields a P:O approaching 1.0 with NADH₂; it is uncoupled by pentachlorophenol but not by dinitrophenol. The soluble fraction catalyzes pentachlorophenol-sensitive ATP-Pi³² exchange and ATPase activity, as well as an ATP-ADP exchange which followed recoupling activity during purification.

SUCCINATE-LINKED REDUCTION OF NAD; ACTIVATION OF SUCCINATE OXIDATION

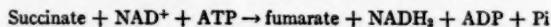
These properties of the respiratory chain, related to reversal of oxidative phosphorylation, have been intensively studied, particularly in the laboratories of Klingenberg, Chance, and Ernster. There is relatively little disagreement with respect to the actual observations, but the interpretation of these effects in terms of mechanisms has provoked some controversy.

Klingenberg & Bücher have reviewed the background of this problem (13). Klingenberg *et al.* (168, 169) found that NAD of aerobic mitochondria from many different tissues is almost entirely in the oxidized form. Surprisingly only a small fraction of mitochondrial NAD could be reduced by NAD-linked substrates in the controlled state (lack of ADP), indicating that it is largely bound or unavailable, perhaps by compartmentation. However, mitochondrial NAD underwent reduction (80% or more) when succinate or α -glycerophosphate was added; the latter substances are of course not able to reduce NAD directly in mitochondria. This effect was seen in all types of mitochondria tested and was considered to be caused by reversal of oxidative

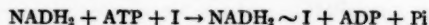
phosphorylation, since it is blocked by DNP and Ca^{++} . In accordance with this view, Klingenberg & Schollmeyer (170) demonstrated that ATP inhibited respiration in mitochondria oxidizing α -glycerophosphate; the decrease in respiration was accompanied by an increase in reduction of mitochondria NAD^+ . Inhibition by ATP did not occur in the presence of uncoupling agents, and could not be demonstrated without ethylenediamine tetraacetic acid in the medium; Mg^{++} addition abolished the effect. Addition of ATP was also found to shift the oxidation-reduction state of flavoprotein and of cytochrome-*b*, which became more reduced, and cytochrome-*c* and -*a*, which became more oxidized, an effect which Klingenberg & Schollmeyer showed (171) to be greatly potentiated by addition of the NAD linked electron acceptor oxalacetate to "pull" electrons. Thus, by driving the reversal of the coupling mechanisms, ATP can cause a profound shift of the substrate end of the respiratory chain to a more reduced state, at the expense of reducing equivalents from the cytochrome end of the chain.

Chance and his colleagues, who had in fact first observed the effect of succinate on NAD reduction in 1955 (172), also made observations similar to those of Klingenberg and have formulated a mechanism to account for the succinate effect. They found the rate of reduction of mitochondrial pyridine nucleotide by succinate to be only one-tenth the rate of over-all respiration; pyridine nucleotide is thus not an obligatory carrier in succinate oxidation (173). Such NAD reduction was inhibited by malonate and by fumarate, indicating participation of succinic dehydrogenase (174). Amytal and antimycin-A also inhibited the effect, suggesting participation of the (bifurcated) respiratory chain between NAD and succinate (175). Oligomycin and dinitrophenol also inhibited NAD reduction, as did ADP and phosphate, in accordance with the view that reversal of oxidative phosphorylation is driving succinate-linked reduction of NAD (176).

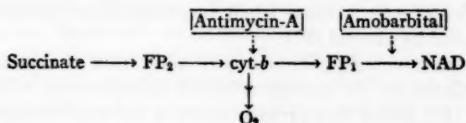
Direct participation of added ATP in the reduction of NAD was demonstrated by Chance using either cytochrome-*c* (177, 178) or succinate (179) as electron donors. It was shown (180, 181) that from 2.0 to 3.0 moles of ATP were required for reduction of one mole of NAD; Chance suggested that the true ratio is 2.0, assuming the overall mechanism of oxidative phosphorylation postulated by Chance & Williams (5). The stoichiometry of reduction of NAD was suggested to be:



a thermodynamically possible reaction (180). Presumably the NADH_2 so formed reacts with a second ATP to form the "inhibited," high-energy form:



Chance & Hollunger have postulated that mitochondrial NAD^+ is reduced by equivalents from succinate traversing the pathway



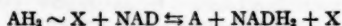
This is the minimum representation of a pathway which contains both the amobarbital- and antimycin-blocked sites; their data do not exclude a longer (bifurcated) chain or participation of ubiquinone (175).

Ernster and his colleagues (57) also studied reduction of NAD by succinate, by measuring the net reduction of acetoacetate to β -hydroxybutyrate (via D- β -hydroxybutyric dehydrogenase) at the expense of succinate in aerobic mitochondria. Acetoacetate reduction occurred under aerobic conditions, but only if phosphate acceptor was absent; it was inhibited by amobarbital, antimycin-A, and dinitrophenol, corresponding to the findings of Chance *et al.* However, cyanide or anaerobiosis also blocked the reduction of acetoacetate by succinate, which would not be expected by the Chance hypothesis. Ernster therefore concluded that the oxidation of succinate by oxygen, which can be blocked by antimycin-A or cyanide, but not amobarbital, is a necessary event for the reduction of acetoacetate at the expense of succinate. Succinate oxidation yields high-energy intermediates postulated to be necessary to drive electrons from succinate back to acetoacetate via mitochondrial NAD: these cannot be replaced by added ATP. Ernster therefore holds that the path of electrons from succinate to acetoacetate involves the amobarbital-sensitive site but not the antimycin-sensitive step; it is the air oxidation of succinate which is blocked by antimycin. This conception of the mechanism of succinate-linked reduction of NAD incorporates an explanation of another phenomenon not readily accounted for by the other hypotheses, namely the activation of succinate oxidation by ATP, to be discussed below.

Krebs and his colleagues (182 to 185) also studied the reduction of acetoacetate by succinate in tissue homogenates, but found no evidence whatsoever of an amobarbital or antimycin inhibition. They therefore concluded it is unnecessary to postulate a reversal of oxidative phosphorylation to account for acetoacetate reduction by succinate, and proposed that when succinate is present its oxidation "monopolizes" the respiratory chain so that NADH_2 is not oxidized by the chain, but by the alternative oxidant acetoacetate, leading to accumulation of β -hydroxybutyrate. More recently Ernster (57) has presented data which apparently resolve the very puzzling discrepancy between the two laboratories with regard to the effect of amytal. He has found that acetoacetate reduction in the presence of succinate is not inhibited by amytal in whole homogenates in confirmation of Krebs *et al.*, but it is inhibited in isolated mitochondria. Ernster showed that the soluble fraction of liver contains other reducing systems which are available for

direct reduction of acetoacetate when succinate-linked reduction of acetoacetate is blocked by amytal (57).

The newly discovered phenomenon of activation of succinate oxidation by ATP is related to the succinate-linked reduction of NAD. Azzone & Ernster (186, 187) found that preincubation of rat liver mitochondria in the presence of arsenate and bishydroxycoumarin before addition of succinate depressed the rate of oxygen uptake with succinate to less than one-tenth its normal rate. Addition of ATP restored oxidation of succinate; it could not be replaced by cytidine triphosphate, uridine triphosphate, inosine triphosphate, and guanidine triphosphate, or by adenosine monophosphate, ethylenediamine tetraacetate, cysteine sulfinate, or increased succinate concentration. Azzone & Ernster concluded that succinate oxidation requires ATP or other high-energy intermediates which are depleted by the preincubation. They suggest that in phosphorylating oxidation of succinate in intact mitochondria, electron transport must go via a thermodynamically unfavorable step involving a carrier (suggested to be the NAD-linked flavoprotein) which can be reduced by succinate only with input of energy. When this high-energy, reduced carrier is oxidized by the next carrier, presumably cytochrome-*b*, the high-energy intermediate fed into the system as "primer" is recovered; the ensuing electron transport from cytochrome-*b* to oxygen then yields a net of two phosphorylations for oxidation of succinate. Azzone *et al.* (188) and Ernster (57) suggest that the requirement of ATP for succinate oxidation is related to succinate-linked reduction of NAD, in that the ATP-activated carrier formed by succinate dehydrogenation may either be utilized for reducing NAD⁺, as follows:



or may undergo oxidation by cytochrome-*b*:



Inactivation of succinate oxidation by depletion of high-energy intermediates and reactivation by ATP and ATP-producing systems has also been independently observed by Williams (189), Chance & Hagihara (190), and Klingenberg & Schollmeyer (191). However, the interpretation offered by Ernster and his colleagues has been questioned. An alternative explanation is that ATP removes oxalacetate accumulating during the preincubation. Oxalacetate is a potent inhibitor of succinate oxidation; ATP is known to abolish such inhibition [Pardee & Potter (192)] in an enzymatic reaction in which oxalacetate does not disappear [Tyler (193)]. Ernster *et al.* (57) reject this mechanism, on the basis that cysteine sulfinate did not remove the inhibition (i.e., by transamination with oxalacetate), that no more than $5 \times 10^{-8} M$ oxalacetate could be detected, and that guanidine triphosphate and inosine triphosphate, preferred by oxalacetate carboxylase to ATP, were much less active than ATP in activating succinate oxidation. Scholl-

meyer & Klingenberg (194) found that oxalacetate is present in sufficient concentrations in "depleted" mitochondria to be inhibitory to succinate oxidation. However, they showed that ATP does not cause extensive removal of oxalacetate although it activates succinate oxidation; on the other hand cysteine sulfinate which, they showed by direct analysis, removed oxalacetate very effectively, did not reactivate succinate oxidation. These findings thus support Ernster's hypothesis. Klingenberg & Schollmeyer (191) also found that succinate oxidation and succinate-linked NAD reduction go together; the former appears to be a prerequisite for the latter. Much further work is required to rationalize all the findings, which present new and possibly revealing aspects of the mechanisms of oxidative phosphorylation.

Attempts have been made in a number of laboratories to detect the reduction of added NAD by succinate and ATP in submitochondrial systems. Löw *et al.* (195) observed increased absorption at 340 $m\mu$ on addition of ATP, NAD⁺, and succinate to anaerobic submitochondrial heart particles. The effect requires ATP and is inhibited by ADP and by phosphate, and also by oligomycin, amobarbital, and DNP, although the latter is not completely effective. It was concluded that net reduction of added NAD⁺ had taken place. Gregg & Lehninger made similar observations with sonic particles from rat liver (196). However, they found that most of the increase in absorption at 340 $m\mu$ occurring with ATP, NAD, and succinate is caused by very pronounced light-scattering changes in the particles specifically induced by these components, which were prevented by amobarbital and DNP. They concluded that succinate and ATP are specific in driving conformational changes in the mitochondrial membranes (cf. 10). Net reduction of added NAD⁺ by succinate apparently can occur in submitochondrial systems but is dependent on still unknown factors.

An independent lead to the problem is presented by recent work of Wise & Lehninger (197), who found that the bound D- β -hydroxybutyric dehydrogenase of sonic particles of rat liver is protected from reversible aerobic inactivation by the combination of succinate, ATP, and NAD⁺, but not by NAD-linked substrates such as β -hydroxybutyrate. Aerobic inactivation in the absence of these components could be reversed by incubation with 2,3-dimercaptopropanol or other thiols, in the presence of succinate and NAD⁺. Succinate is therefore specific for maintaining the bound NAD of mitochondrial β -hydroxybutyric dehydrogenase in the stable reduced state.

OXIDATIVE PHOSPHORYLATION AND MECHANOCHEMICAL CHANGES IN MITOCHONDRIAL MEMBRANES

Water uptake and extrusion in mitochondria are the result of changes in the structure of the membranes which are directly dependent on intermediate reactions of oxidative phosphorylation [see Lehninger (10)]. "Active" ion movements in mitochondria are also dependent on coupled respiration [cf. Bartley & Davies (198), Mitchell (62), Robertson (9)]. Both the mechano-

chemical (196) and ion fixation (199, 200) activities can be observed in fragments of the membranes; both require coupled electron transport and may be blocked by reagents such as azide. These and other findings support the hypothesis of Lehninger (10, 60, 61) that transformation of respiratory energy is trimodal. This conception suggests that the mechanism of oxidative phosphorylation should reveal something of the mechanism of transport and mechanochemical activities and, conversely, that study of the membrane changes and active transport should shed light on oxidative phosphorylation. The finding that C-factor, a protein involved in mitochondrial contraction, can also recouple oxidative phosphorylation is an example (see above).

Work in the past few years (cf. 10) has shown that water uptake by isolated mitochondria is inhibited by anaerobiosis, cyanide, amobarbital, and antimycin-A, indicating that respiration is required for the increase in permeability, particularly if ADP is lacking. This process is greatly stimulated by the so-called swelling agents, which include phosphate, thyroxine, Ca^{++} , phloridzin, glutathione, and higher fatty acids, among others (cf. 10). That intermediate reactions of oxidative phosphorylation are also required is shown by the inhibition of swelling by DNP, and by the recent observation that oligomycin (201) and potassium atractylate (91) also inhibit this process.

Water is extruded from swollen mitochondria when phosphorylating respiration is induced in the presence of phosphate acceptor, or on addition of ATP when respiration is blocked by anaerobiosis or cyanide (202). Contraction is not inhibited by DNP or bishydroxycoumarin when ample ATP is present. On the other hand it is blocked by azide (202), and by sucrose and a wide variety of other polyhydroxylic compounds which also block the adenosine triphosphatase linked to oxidative phosphorylation (95). Recently further evidence for a close relationship between mitochondrial contraction and the coupling mechanism was given by the finding of Vignais *et al.* (91) that atractylate blocks ATP-linked contraction and of Neubert & Lehninger (203) that oligomycin also prevents this process. The swelling and contraction of mitochondria are relatively independent of ionic constituents of the medium and their concentration, ruling out ion fluxes driven by respiration as the basis of the water movements. In fact swollen mitochondrial "ghosts" unable to respire or to fix K^+ are still capable of water extrusion when ATP is added (204).

When mitochondria are swollen by reduced glutathione they do not contract on the addition of ATP (205); glutathione causes detachment of a necessary protein factor (C-factor) from the mitochondria into the medium (144, 145). Purified C-factor increases the P:O ratio of digitonin fragments, as described above, indicating that it is involved in both the mechanochemical and phosphorylative activities of the membrane.

Lehninger postulated that one or more of the intermediate enzymes of oxidative phosphorylation may be "mechanoenzymes" capable of conformational changes depending on their energized state in the coupling mechanism

(10, 61, 202). Changes in conformation of protein or enzyme molecules depending on enzymatic or oxidation-reduction state are being observed increasingly, as in the cases of cytochrome-*c*, hemoglobin, flavoproteins, and of course the actomyosin system; Koshland's "induced fit" theory of enzyme action also postulates conformational changes (206). Such changes have recently been reviewed by Okunuki (207). This conception that the coupling enzymes are integral with the structures causing permeability and conformational changes in the membranes is supported by the striking similarities between myosin ATPase and mitochondrial ATPase activities (8, 10, 61, 76, 83, 84, 112). These similarities include the biphasic role of -SH groups (76, 111, 112), the stimulation of both ATPases by DNP (cf. 208), and the catalysis of the rather unique $\text{H}_2\text{O}^{18}\text{-Pi}$ exchange reaction (209), among others. The multistep nature of both ATPase systems is especially noteworthy; and studies of mitochondrial ATPase, especially the soluble enzyme of Pullman *et al.* (83, 84), should benefit from contemplation of the complexities of the myosin ATPase activity. Finally, the relationship of mechanical and enzymatic events may now be studied in small sonic fragments of the membrane and should yield new clues (196).

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CHEMISTRY OF THE CARBOHYDRATES¹

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The emphasis in last year's *Review* (1) was on the chemistry of monosaccharides. This review is restricted largely to the structural chemistry of polysaccharides and of certain other carbohydrate-containing natural polymers. Although some of the topics to be discussed here have not been reviewed for several years, the literature survey is intended to cover the past two years only, reference being made to monographs and other reviews for previous work.

A special issue of *Bulletin de la Société de Chimie Biologique* has reported the proceedings of an international colloquium on the biochemistry of carbohydrates held in July 1960 at Gif-en-Yvette; a wide variety of topics was reviewed. A monograph by Gottschalk (2) surveys the chemistry and biology of sialic acids and related substances. Biochemical Society Symposium No. 20 (3) contains reviews of the following topics: chondroitin sulphates and sulphated polysaccharides of connective tissue (H. Muir), the histological demonstration of connective-tissue mucopolysaccharides (R. C. Curran), the analysis of mucopolysaccharides (S. Gardell), the structure and function of hyaluronate (H. J. Rogers), nucleotides in the interconversion of sugars and in glycoside synthesis (I. D. E. Storey), some biochemical aspects of sulphated mucosubstances (P. W. Kent), and the enzymic degradation of mucopolysaccharides (P. G. Walker). Other reviews have discussed the stereochemistry of sugars (4), brain gangliosides (5), 3,6-dideoxyhexoses (6), aminopolysaccharides (7), wood polysaccharides (8), the biochemistry of starch and glycogen (9), alkaline degradation of cellulose and oxidized celluloses (10), long-chain derivatives of monosaccharides and oligosaccharides (11), oxidations with oxygen and platinum catalysts in carbohydrate chemistry (12), and the structure and immunological specificity of polysaccharides (13).

GENERAL METHODS

Chromatographic procedures are being extended to the separation of carbohydrates of higher molecular weight and especially of polysaccharides. The rapid fractionation of neutral monosaccharides (14) and oligosaccharides (15) according to molecular size may be achieved using the neutral salt forms of sulphonic acid-type ion exchangers. Chondrosin and hyalobiouronic acid may be separated on a cation exchange resin (16). Acid mucopolysaccharides have been separated on the basis of the differential solubility of their cetylpyridinium complexes followed by chromatography on an anion exchange resin (17). Ion-exchange celluloses are finding widespread applica-

¹ The survey of literature pertaining to this review was completed in August 1961.

tion in the fractionation of polysaccharides for analytical and preparative purposes. Neutral polysaccharides are eluted from diethylaminoethyl (DEAE)-cellulose at pH 6 with buffers of increasing strength, whereas acidic polysaccharides are only eluted at a higher pH (18). Diethylaminoethyl cellulose has been used for the fractionation of pectic substances (18), nonstarchy polysaccharides and glycoproteins from wheat flour (19), and seromucoids from human serum (20). Hyaluronic acid, chondroitin 4-sulphate, and heparin may be separated on epichlorohydrintriethanolamine (ECTEOLA-) cellulose (21), and the procedure has been used to separate acid polysaccharides from mast cell tumors in mice (22).

Methods for the structural modification of polysaccharides involving selective reduction of carboxyl groups and their formation by the selective oxidation of hydroxymethyl groups were reviewed last year (1). Carboxyl-reduced chondroitin has been prepared from chondroitin 4-sulphate by successive treatments with methanolic hydrogen chloride and sodium borohydride (23). The preparation of carboxyl-reduced heparin involves the reduction of acetylated heparin with diborane (24).

A number of modified amyloses and celluloses have been prepared using reactions well known in monosaccharide chemistry. Selective toluene-*p*-sulphonylation is the key reaction in the preparation of 6-deoxyamylose (25), 6-deoxycellulose (26), and 3,6-anhydroamylose (25, 27). The introduction of 2-deoxy-D-glucose residues into cellulose has been achieved (28) by the reduction of a cyanoethyl cellulose (29) with sodium in liquid ammonia. Amylose has been converted into a glucose-altrose polymer (30) by the treatment of 6-*O*-trityl-mono-*O*-tosyl amylose with alkali followed by detritylation.

Water-soluble polystyrene-sulphonic acid provides a convenient catalyst for the controlled fragmentation of labile polysaccharides (31), especially when continuous dialysis is used to remove oligosaccharides (32). This acid acts as a selective catalyst in the acid hydrolysis of basic polysaccharides (33).

HEMICELLULOSES

The very considerable activity which has been shown in this field during recent years (34) has continued unabated. In the absence of strict definitions, the term "hemicellulose" will be taken to include those polysaccharides of plant origin which contain basal chains of D-xylose, D-mannose, D-mannose, and D-glucose, or D-galactose residues to which other sugar residues, often in substantial proportions, may be attached as side chains. Polysaccharides of the same general types as those found in the common woods, cereals, and grasses, occur in ferns (35), in the ancient gymnosperm *Ginkgo biloba* (the maidenhair tree) (36), and in other ancient woods (37).

Xylans.—Xylans carrying single unit 4-*O*-methyl- α -D-glucopyranosyluronic acid side chains attached to position 2 of 1,4-linked β -D-xylopyranose residues in the main chains have been isolated from yellow birch (38), trembling aspen (39), the wood (40) and the bark (41) of white birch, and

the seed hairs of kapok (42). Contrary to an earlier report (43), trembling aspen xylan does not contain L-arabinofuranose residues (39). Xylans containing rather higher proportions of 4-O-methyl-D-glucuronic acid together with some L-arabinofuranose residues as single unit side chains occur in eastern white pine (44), Sitka spruce (45), Scots pine (46), maritime pine (47), tamarack (48), and *Ginkgo biloba* (49) woods. A xylan with L-arabinofuranose residues forming the main type of side-chain units with smaller proportions of D-glucuronic acid and 4-O-methyl-D-glucuronic acid side chains is present in cocksfoot grass (50). The xylans from soybean hulls (51) and maize germ (52) have more complex side chains which contain end groups of D-glucuronic acid, D-galactopyranose, and L-arabinofuranose residues, together with nonterminal L-arabinofuranose residues.

Partly acetylated xylans have been isolated from birch woods (40, 53) by extraction with dimethyl sulphoxide. The most satisfactory procedure for assessing the distribution of acetyl groups, which have been located exclusively on D-xylopyranose residues, and predominantly at C₂, employs phenylisocyanate as a reagent for the protection of hydroxyl groups which are not acylated in the polysaccharide (54).

The selective oxidation of some of the terminal L-arabinofuranose residues in rye flour arabinoxylan followed by the characterization of the aldobionuronic acid, 3-O-(L-arabinofuranosyluronic acid)-D-xylose, formed on graded hydrolysis of the oxidized polysaccharide provides evidence as to the mode of attachment of L-arabinofuranosyl side chains in the polysaccharide (55). In an alternative approach to the same structural problem, the trisaccharide, O-L-arabinofuranosyl-(1→3)-O-D-xylopyranosyl-(1→4)-O-D-xylopyranose (56), has been isolated on enzymic hydrolysis of the arabinoxylans from rye flour and cocksfoot grass (57).

The distribution of L-arabinofuranose side chains along the main chain of the highly branched wheat-flour arabinoxylan (58) has been assessed, using a modification of Barry's procedure for the degradation of periodate-oxidized polysaccharides (59). Since only those xylose residues which carry side chains are resistant to cleavage by periodate, the isolation of xylose, xylobiose, only small amounts of xylotriose, but no fragments of higher molecular size shows that side chains are attached to not more than three, and most frequently to not more than two contiguous, xylose residues. Similar degradations using F. Smith's procedure (60), namely, reduction of the periodate-oxidized polysaccharide, followed by controlled hydrolysis of the derived polyalcohol, have been carried out on the hemicelluloses of brome grass (61) and oat hulls (62). The isolation in this way of O-β-D-xylopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→2)-glyceritol from oat hull hemicellulose (62) provides the first conclusive evidence that small proportions of D-glucose residues may be integral constituents of polysaccharides of the xylan group.

The degradation of xylans with cold dilute alkali under conditions often employed for their isolation from the plant has been studied (63); it is reported that degradation is slow and proceeds slowly from the reducing

group. A mechanism has been proposed for the "by-passing" of branching points during the alkaline degradation of 1→4-linked polysaccharides which carry side chains attached to position 3 (63). The mechanism is supported by the observation that both methanol and D-xylose are eliminated when 3-O-methyl-4-O-(β-D-xylopyranosyl)-D-xylose is degraded with alkali (64). The mechanism by which 4-O-methyl-D-glucuronic acid residues are cleaved from acidic xylans during alkaline pulping is not fully understood (65).

Mannans, galactomannans, and glucomannans.—In contrast to the galactomannan found in Kentucky coffee beans (66), a mannan composed almost exclusively of 1→4-linked β-D-mannopyranose residues has been isolated from green coffee beans (67). A galactomannan from the kernel of the green palmyra palm nut (68) is of the guaran type, whilst the galactomannan from coconut kernel (69) has some of the same structural features but also contains galactose residues in 1→4 linkages and possibly also as branching points. Structural studies have shown that the glucomannans from white spruce (70), eastern white pine (71), tamarack (72), and *Ginkgo biloba* (73) woods are similar to other glucomannans from coniferous woods (34) in possessing essentially linear chains of 1,4-linked β-D-glucopyranose and β-D-mannopyranose residues in the approximate ratio of 1:3. Glucomannans containing the same general basal structures but carrying some D-galactopyranose end groups have been isolated from southern pine (74), jack pine (75), Norwegian spruce (76), and eastern hemlock (77) woods. Although these polysaccharides are apparently homogeneous, definite evidence that they are galactoglucomannans rather than mixtures of glucomannans and galactomannans is not yet available. The mode of attachment of D-galactopyranose end groups in Norwegian spruce glucomannan has been established by the isolation of O-D-galactopyranosyl-(1→6)-O-β-D-mannopyranosyl-(1→4)-D-mannopyranose as a partial hydrolysis product (76). From the isolation also of four of the six possible trisaccharides containing glucose or mannose residues, or both, it has been suggested that this polysaccharide contains a random distribution of sugar residues in the main chains. Similarly constituted glucomannans, but with relatively higher proportions of glucose residues, occur in much smaller amounts in hardwoods (78, 79).

The presence of small proportions of xylose residues in samples of glucomannans is often attributed to incomplete separation from accompanying xylans. That this is not necessarily the case has been demonstrated by Perila & Bishop (80), who have isolated 6-O-α-D-xylopyranosyl-D-glucopyranose and O-α-D-xylopyranosyl-(1→6)-O-β-D-glucopyranosyl-(1→4)-D-glucopyranose from the enzymic hydrolysis of jack pine glucomannan.

Galactans and arabinogalactans.—The relatively high proportion of galactose residues in compression wood originates from galactans of the type found in certain pectic complexes (81) which are composed of linear chains of 1,4-linked β-D-galactopyranose residues. Such a galactan, which also contains 13 per cent of galacturonic acid residues, is found in Norwegian spruce compression wood where it is associated with an acidic polysaccharide of the pectic acid type (82). Galactans of this type may often occur as minor poly-

saccharide components of woods, and their presence in the normal woods from white birch (83) and Norwegian spruce (76) has been indicated by the isolation of 4-*O*- β -D-galactopyranosyl-D-galactopyranose on partial acid hydrolysis of the wood or of one of its hemicellulose fractions. Another polysaccharide which contains a high proportion of 1,4-linked β -D-galactopyranose residues together with some mannose and arabinose residues occurs in *Centrosema* seeds (84).

The water-soluble arabinogalactans from various coniferous woods possess common structural features in highly branched skeletons of β -D-galactopyranose residues mutually joined by 1 \rightarrow 3 and 1 \rightarrow 6 linkages to which arabinose residues may be attached in the form of L-arabinofuranose or 3-*O*- β -L-arabinopyranosyl-L-arabinofuranose units (34). Arabinogalactans A and B may be isolated from several species of larch wood (85), whereas the arabinogalactans from tamarack (86, 87), maple sap (88), and Monterey pine (89) are apparently homogeneous. Examination of arabinogalactans A and B has shown that the two polysaccharides are very similar in detailed structure (90, 91). The degraded polysaccharides obtained from degradation of the periodate-oxidized arabinogalactans A and B consist largely of main chains of D-galactopyranose residues joined by 1 \rightarrow 3 linkages indicating that the 1,6-linked residues are located in the outer chains of the original polysaccharides (92). Catalytic oxidation of European larch arabinogalactan followed by graded acid hydrolysis of the oxidized polysaccharide furnishes two aldobiouronic acids, 6-*O*-(D-galactopyranosyluronic acid)-D-galactose and 6-*O*-(L-arabinofuranosyluronic acid)-D-galactose (93). The characterization of the latter disaccharide provides evidence for the mode of attachment in the polysaccharide of L-arabinofuranose to D-galactopyranose residues, most probably to those in the main 1 \rightarrow 3-linked chains.

α -GLUCANS

Biosynthesis.—It is now considered that glycogen (94) and starch (95) are normally formed from uridine diphosphate glucose by the action of "synthetases." Phosphorylase is regarded primarily as a degradative agent. Illingworth, Brown & Cori (96) have shown that highly purified muscle phosphorylase-*a* will catalyze the formation of amylose with chain lengths of up to 2000 from α -D-glucose-1-phosphate in the absence of a carbohydrate primer after an initial induction period; this reaction is termed *de novo* synthesis. In the presence of added, highly purified amylo-1,4 \rightarrow 1,6-transglycosylase the rate of polysaccharide formation is enormously increased, and the product is glycogen with unit chain length 13 to 16 and with molecular weight 2 to 25 $\times 10^6$. From a study of the mechanism of the reaction there is evidence that amylose chains are attached to the enzyme protein rather than to one of the functional groups of the coenzyme (97). This type of synthesis of glycogen could be of special importance during embryonal development.

Amylose.—As indicated in an earlier review (98), barriers to the complete degradation of amylose by β -amylase and phosphorylase may be introduced artificially during isolation. It is believed, however, that there are some

"natural" barriers in amylose. Such barriers to complete β -amylolysis are not due to the presence of phosphate esters (99). Greenwood (100) has sub-fractionated potato amylose by Everett & Foster's method (101); the hydrodynamic properties of some of these subfractions suggest that a small degree of branching may occur in some amylose molecules. Banks, Greenwood & Jones (102) have demonstrated that the hydrolytic action of Z-enzyme, which removes barriers to the complete amylolysis of some amyloses, is indistinguishable from that of an α -amylase in its action on amylose and amylopectin. The same conclusion has been reached by Cunningham, Manners, Wright & Fleming (103).

Amylopectin.—Amylopectins and glycogens differ markedly in their physical behavior, and the measurement of limiting viscosity and of the concentration-dependence of the sedimentation coefficient serves to classify polysaccharides whose unit chain lengths are intermediate between those of typical glycogens and amylopectins (104). Despite the low value of 15 for the unit chain length of anomalous amylopectins from potato and rubber seed starches (105), these polysaccharides are considered to be degraded amylopectins rather than glycogens. The "36-unit" amylopectins of high amylose starches from wrinkled-seeded peas and certain varieties of maize have been shown to be artefacts arising from the presence of contaminating, short-chain amylose in normal amylopectins (106). Further studies (107, 108) on barley starches, isolated before and after malting, have confirmed and extended previous results (109), and it is suggested that malt amylopectin has undergone limited β -amylolysis, whilst malt amylose has been partially degraded by α -amylase. The action of various amylases on starch granules appears to be limited to the accessible surface regions of the polysaccharide components (110, 111).

Glycogen.—A detailed study by Stetten & Katzen of the degradation of glycogen by alkali (112) has confirmed previously reported conclusions (98) that the Pflüger method for the extraction of glycogens with hot alkali results in the formation of degraded polysaccharides of lower molecular weight than those of native glycogens. The alkali-stable glycogens isolated by this method have been shown to be polysaccharide acids in which the reducing group is replaced by an isosaccharinic acid residue. Glycogen, which has been treated with sodium borohydride, is stable to alkali under anaerobic conditions. Stetten & Stetten (113, 113a) have reviewed the abnormalities of glycogens found in diseased storage organs. Most of these abnormalities have been related to the specific impairment of particular enzymes of the glycogen cycle, including glucose-6-phosphatase, phosphorylase, branching and de-branching enzymes; in other cases no biochemical lesions have yet been found. Further examples of storage-disease glycogens have been studied by Manners & Wright (114, 115). Glycogens from pre- and post-phases of *rigor mortis* in mammalian tissues have been examined; in only one case were significant differences found (116).

Isolichenin.—Isolichenin contains 55 per cent of 1,3 and 45 per cent of 1,4 linkages (117). Partial acid hydrolysis of the polysaccharide furnishes

maltose, nigerose, maltotriose, nigerotriose, and the two possible trisaccharides containing mixed linkages; the results indicate that there is a lack of regularity in the distribution of the two types of linkage.

β -GLUCANS

1 \rightarrow 3-Linked β -glucans continue to be recognized as of widespread occurrence. They are elaborated by the unicellular alga *Euglena gracilis* (118) and by marine diatoms (Chrysophyceae) (119). The structure of callose has been further substantiated by the isolation of laminaribiose and laminaritriose as products of enzymic degradation (120). C^{14} -Labelled sucrose is transformed into radioactive callose by germinating pollen (121). Extracts of mung-bean seedlings catalyze the synthesis of a 1 \rightarrow 3-linked β -glucan from uridine diphosphate D-glucose (122).

Two different approaches have been used to assess the distribution of 1 \rightarrow 3 and 1 \rightarrow 4 linkages in the linear β -glucans in cereals, and conflicting conclusions have been reached regarding the fine structure of oat β -glucan. F. Smith and Sorger-Domenigg reduced periodate-oxidized oat β -glucan and from a subsequent controlled-acid hydrolysis they isolated 2-O- β -D-glucopyranosyl-D-erythritol, 2-O- β -laminaribiosyl-D-erythritol, and 2-O- β -laminaritriosyl-D-erythritol (123, 123a). These results point to the presence of blocks of two and three 1 \rightarrow 3-linked glucose residues in parts of the molecular structure, in addition to isolated 1 \rightarrow 3 linkages. Parrish & Perlin (124) have examined the modes of action of "cellulase" and "laminarinase" on polysaccharides with mixed linkages. Cellulase apparently splits bonds in the sequence . . . 4 β -D-Glcp 1 \rightarrow 4 β -D-Glcp 1 . . . , whereas laminarinase cleaves 3-O-substituted β -D-glucopyranosyl linkages rather than the 1 \rightarrow 3 bonds between glucose residues. On the basis of the oligosaccharides isolated from the enzymic degradation of oat and barley β -glucans with laminarinase and cellulose, Parrish, Perlin & Reese (125) conclude that both polysaccharides contain only isolated 1 \rightarrow 3 linkages which are separated by two or three 1 \rightarrow 4 linkages. 1 \rightarrow 2-Linked β -glucans, which are elaborated by various *Argobacteria*, are resistant to complete methylation (126). This resistance to etherification is probably due to hydrogen bonding of the hydroxyl group at C₂ to the ring oxygen (127). Partial hydrolysis and periodate oxidation studies, however, indicate that the polysaccharides contain only 1 \rightarrow 2 linkages (126).

PECTIC SUBSTANCES

The view that pectic substances are complexes of three homopolysaccharides, i.e., araban, galactan, and galacturonan (pectic acid), is being increasingly recognized as an oversimplification. With the exception of the extraction of pectic acid from sunflower heads (128), all recent attempts to prepare pure galacturonans have yielded polysaccharides that contain appreciable proportions of neutral sugar constituents. Fractionation of various fruit pectins via their copper complexes furnishes two different types of polysaccharides, high and low in hexuronic acid content, but com-

posed of the same neutral sugar residues (129). Pectic acids from sugar beet (18) and lucerne (alfalfa) (130), which are chromatographically homogeneous on diethylaminoethylcellulose, still contain L-rhamnose, L-arabinose, D-galactose, and traces of 2-O-methyl-D-xylose and 2-O-methyl-L-fucose as constituent sugars. In a detailed investigation of lucerne pectic acid (130) it has been shown that D-galactose, which is present mainly as end group, and L-rhamnose, which is present in the aldobiouronic acid 2-O-(D-galactopyranosyluronic acid)-L-rhamnose, must be integral components of acidic polysaccharides.

The lability of pectin (pectic acid methyl ester) in alkaline solutions has been attributed to the scission of internal glycosidic linkages by a β -elimination mechanism (131). This type of reaction, which results in the formation of Δ -4,5 unsaturated galacturonic acid end groups, has been shown to take place when pectins are either heated in neutral solution (132) or degraded with certain pectinases (133).

An alkali-stable polysaccharide may be isolated from sugar-beet pectin (134). Although the polysaccharide is composed largely of L-arabinofuranose residues mutually linked as in other arabans (135), significant proportions of residues of D-galactose, L-rhamnose, and galacturonic acid were found to be present, and attempts to isolate a pure araban were unsuccessful.

PLANT GUMS

The comprehensive monograph by Smith & Montgomery (136) gives a detailed account of the chemistry of plant gums and mucilages up to 1958. A detailed study of *Acacia pycnantha* gum (137) has indicated similarities in structure to gum arabic in that the polysaccharide contains a framework of D-galactopyranose residues in which 1 \rightarrow 3 linkages are located largely in the main chains and 1 \rightarrow 6 linkages in the side chains and in the presence of units of L-arabinofuranose and of the aldobiouronic acid, 6-O-(β -D-glucopyranosyluronic acid)-D-galactose, attached as side chains. The gum differs, however, from gum arabic in that 3-O-L-arabinofuranosyl-L-arabinofuranose units replace those of 3-O- α -D-galactopyranosyl-L-arabinofuranose and 3-O- β -L-arabinopyranosyl-L-arabinofuranose in gum arabic, and in the mode of attachment of the various arabinose residues to the main galactan framework. The degraded arabinose-free polysaccharides from *Acacia catechu* (138) and *A. sundra* (139) gums also contain highly branched structures. Jones & Thomas (140) have established that the polysaccharide component of the oleo-resin "gum asafoetida" contains a similar arrangement of D-galactose residues, in the inner chains of the molecular structure, to which are attached side chains terminated by L-arabinofuranose and D-glucuronic acid residues. The D-glucuronic acid residues, which are present in part as the 4-methyl ether, are joined to D-galactose by a 1 \rightarrow 6 linkage.

Another type of plant gum containing inner chains of D-galactopyranose residues which are joined mainly by 1 \rightarrow 6 linkages is exemplified by gum ghatti (from *Anogeissus latifolia*). Partial acid hydrolysis of the gum furnishes two polymer homologous series of oligosaccharides, O- β -D-galactopyr-

anosyl-[(1→6)-*O*-β-D-galactopyranosyl]_n-(1→6)-D-galactose and *O*-β-D-galactopyranosyl-[(1→6)-*O*-β-D-galactopyranosyl]_n-(1→3)-L-arabinose, the latter indicating that L-arabinose residues are present in interior chains as well as in the outer chains (141). The gum from the related species, *Anogeissus schimperi*, contains many similar structural features to those of gum ghatti and partial acid hydrolysis affords the same two polymer homologous series of oligosaccharides (142). The gum, however, contains a higher proportion of D-galactose residues mutually linked by 1→3 linkages and the majority of hexuronic acid residues are accommodated in units of 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose. The partial hydrolysis products of *Albizzia zygia* gum include 3-*O*-β-D-galactopyranosyl-D-galactose, 6-*O*-β-D-galactopyranosyl-D-galactose, 3-*O*-β-D-galactopyranosyl-L-arabinose, 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose (143), but in the absence of further evidence it is not possible to related detailed sequences of sugar units with those of other gums.

Khaya senegalensis gum (144) contains two distinct acidic polysaccharides; the major component is similar to the polysaccharide from *Khaya grandifolia* gum (145) in containing main chains of 1,4-linked D-galacturonic acid and 1,2-linked L-rhamnose residues, the latter carrying through C₄ side chains of D-galactopyranose and 4-*O*-(4-*O*-methyl-D-glucopyranosyluronic acid)-D-galactose units.

ALGAL POLYSACCHARIDES

In contrast to the 1→4-linked xylans from land plants (34) and to the xylan from the red seaweed *Rhodomenia palmata* (146) which contains 1→3 and 1→4 linkages, the xylan from the green seaweed *Caulerpa filiformis* elaborates a xylan which is composed entirely of 1→3-linked β-D-xylopyranose residues (147). The floridean starch from *Dilsea edulis* has been re-examined (104, 148, 149). Values of 15 and 18 for the unit chain length indicate that the polysaccharide is intermediate between typical glycogens and amylopectins, but the iodine-binding power of the polysaccharide (104) and its partial degradation with R-enzyme (149) serve to classify it as an amylopectin. The limiting viscosity number and the concentration dependence of the sedimentation coefficient of floridean starch point unambiguously to the amylopectin class (104). Further structural complexities, however, are indicated by the isolation of nigerose from both partial acid hydrolysis and enzymic hydrolysis using β-amylase and R-enzyme or α-amylase and R-enzyme (148, 149). A glucan of the amylopectin type has been isolated from *Caulerpa filiformis* (150).

Agarose, the principal polysaccharide component of agar, is composed of units of agarobiose, 4-*O*-(β-D-galactopyranosyl)-3,6-anhydro-L-galactose, mutually joined by 1→3 linkages (151). A negligible degree of branching in agarose from *Gelidium amansii* is shown by the isolation of derivatives of 2-*O*-methyl-3,6-anhydro-L-galactose and 2,4,6-tri-*O*-methyl-D-galactose with only traces of tetra-*O*-methyl-D-galactose on exhaustive methanolysis of methylated agarose (152), and the nature of the glycosidic linkages is

confirmed by the isolation in 82 per cent yield of derivatives of 2,4,6-tri-*O*-methyl- β -D-galactopyranosyl-(1 \rightarrow 4)-2-*O*-methyl-3,6-anhydro-L-galactose on partial methanolysis (153).

Carrageenin from *Chondrus crispus* and related red seaweeds contains two definite polysaccharide components (151). The main feature of κ -carrageenin is the presence of alternating sugar units joined by the same types of linkage as in agarose, D-galactose residues carrying sulphate esters at C₄ and 3,6-anhydro-L-galactose residues being replaced by those of the D-isomer (151). λ -Carrageenin is a sulphated galactan; linkage analysis studies have shown that most, if not all, of the linkages are 1 \rightarrow 3 (154). When commercial λ -carrageenin is treated with dilute alkali the disappearance of galactose 6-sulphate residues is accompanied by the appearance of 3,6-anhydrogalactose residues, and it has been suggested by Rees (155) that carrageenin may contain a third polysaccharide component which is the precursor of κ -carrageenin. The red alga *Furcellaria fastigata* contains a polysaccharide which is very similar to κ -carrageenin. Methylation studies have shown that D-galactose residues are involved in 1 \rightarrow 3 and 1 \rightarrow 6 linkages (156), and the diethyl mercaptal of carobiose, 4-*O*-(β -D-galactopyranosyl)-3,6-anhydro-D-galactose, is one of the products of partial mercaptolysis of the polysaccharide (157).

Porphyrin, a sulphated galactan from *Porphyra umbilicalis* (158), is very similar to the galactan from *Porphyra cadensis* (159) in containing residues of D- and L-galactose, 3,6-anhydro-L-galactose, and 6-*O*-methyl-D-galactose. Much of the ester sulphate is accounted for by the isolation of L-galactose 6-sulphate on mild acid hydrolysis of the polysaccharide (160). The seaweed itself contains a sulphatase (161, 162), which either by itself or in close association with other enzymes catalyzes the formation of 3,6-anhydro-L-galactose from L-galactose 6-sulphate residues in porphyran (163).

The green seaweeds contain extremely complex mixtures of polysaccharides (164). Further investigations (165) have shown that the water-soluble polysaccharides from *Cladophora rupestris* carry sulphate esters of sugar residues (namely, arabinose or rhamnose) other than galactose (165). Rhamnose residues are sulphated in the water-soluble polysaccharides from *Enteromorpha compressa* (166). The water-soluble polysaccharides from *Acrosiphonia centralis* include a starch-type glucan and a sulphated polysaccharide containing residues of D-xylose, L-rhamnose, and D-glucuronic acid [cf. *Ulva lactuca* (167)], which affords the aldobiouronic acid, 4-*O*-(β -D-glucopyranosyluronic acid)-L-rhamnose, on graded hydrolysis (168).

GLYCOSAMINOGLYCURONOLYCANES (ACIDIC MUCOPOLYSACCHARIDES)

A revised system of nomenclature for mucopolysaccharides has been proposed by Jeanloz (169). The glycosaminoglycuronoglycans of connective tissue are now recognized as forming a family of related polysaccharides in which alternating hexuronic acid residues are linked by 1 \rightarrow 3 linkages to hexosamine residues which are joined by 1 \rightarrow 4 linkages to hexuronic acid.

Hyaluronidases.—Marked differences are found in the specificities of

hyaluronidases from various sources. Testicular hyaluronidase is an endo-*N*-acetylhexosaminidase which acts on hyaluronic acid, chondroitin 4- and 6-sulphates, but not on dermatan sulphate or heparin (170). In the case of hyaluronic acid, the major end-product is a tetrasaccharide, although all members of a regular series of oligosaccharides ranging from di- to tetradecasaccharides in increments of one disaccharide unit may be isolated. In contrast, leech hyaluronidase is an endohexuronidase which furnishes a tetrasaccharide as the principal product from hyaluronic acid, but is without action on chondroitin or its sulphates (171). With testicular and leech hyaluronidases and from the subsequent action of dilute alkali or of liver exo- β -glucuronidase or exo- β -glucosaminidase it is possible to prepare hyaluronate oligosaccharides with any combination of glucuronic acid and glucosamine as reducing and nonreducing end groups (171). The mode of linkage of glucuronic acid to glucosamine residues in hyaluronic acid follows from studies on the trisaccharide, *O*- β -D-glucosaminyl-(1 \rightarrow 3)-*O*-(β -D-glucopyranosyluronic acid)-(1 \rightarrow 4)-D-glucosamine, which is formed from the testicular tetrasaccharide by the action of liver β -glucuronidase (172). Methylation of the trisaccharide, followed by esterification, reduction with lithium aluminium hydride, and hydrolysis, gives 2,3-di-*O*-methyl-D-glucose together with methyl ethers of glucosamine.

Whilst testicular and leech hyaluronidases are normal glycosidases, bacterial hyaluronidases degrade hyaluronic acid and chondroitin with cleavage of the hexosaminic bond with the formation of Δ 4,5 unsaturation in the hexuronic acid residue. Thus 3-*O*-(β -D-4,5-glucoseenpyranosyluronic acid)-*N*-acetyl-D-glucosamine is formed from hyaluronic acid, the site of unsaturation pointing to cleavage of a 1 \rightarrow 4 bond (173). The site of cleavage of the glycosidic bond during the formation of unsaturated oligosaccharides has been localized by carrying out the hydrolysis in O^{18} -labelled water, no O^{18} being incorporated into the product (174). These results, taken together with the earlier observation that tritium-labelled hyaluronic acid releases tritium into the medium (175), establishes the reaction as a β -elimination. Like other glycosidases (176), testicular hyaluronidase cleaves the glycosyl-oxygen bond (174).

Sulphated polysaccharides are degraded by the chondroitinase from *Proteus vulgaris* (177) and by adaptive enzymes from *Flavobacterium* (178) with the formation of unsaturated oligosaccharides with and without sulphate ester groups. The characterization of these substances provides supporting evidence for the structures that have been advanced for the chondroitin sulphates and dermatan sulphate.

Chondroitin sulphates and dermatan sulphate.—A structure may be advanced for chondroitin sulphate A which may now be named chondroitin 4-sulphate (169). Earlier studies (179) led to the characterization of the aldobiouronic acid, chondrosine, as 3-*O*-(β -D-glucopyranosyluronic acid)-D-galactosamine. Since Stoffyn & Jeanloz (180) have characterized 6-*O*-methyl-D-galactosamine as a cleavage product from methylated chondroitin sulphate A, it follows that sulphate ester groups are attached to C₄ of D-

galactosamine residues. This assignment of the position of sulphate ester groups which was indicated by infrared absorption studies (181, 182) is supported by the isolation of the 4-sulphate of *N*-acetyl-D-galactosamine (183) and the unsaturated aldobiouronic acid, 3-*O*-(β -D-4,5-glucoseenpyranosyluronic acid)-*N*-acetyl-D-galactosamine (184) as degradation products. The attachment of *N*-acetyl-D-galactosamine to D-glucuronic acid residues by 1 \rightarrow 4 linkages, which is indicated by the formation of unsaturated oligosaccharides as enzymic degradation products, is confirmed by methylation studies in which it was shown that reduction of the methylated polysaccharides followed by hydrolysis led to the isolation of 2,3-di-*O*-methyl-D-glucose (180).

The structure of chondroitin sulphate C as the isomeric 6-sulphate is less firmly established. The formation of degradation products similar to those from the 4-sulphate indicates that the two polysaccharides contain the same glycosidic linkages (185, 186). The most probable site for the location of sulphate ester groups is at C₆ of *N*-acetyl-D-galactosamine residues, and this assignment is supported by the similarity of the infrared absorptions of chondroitin sulphate C and of the chondroitin sulphate D from shark cartilage which affords *N*-acetyl-D-galactosamine 6-sulphate as a degradation product (183).

Shark cartilage chondroitin sulphate D contains an unusually high proportion of sulphate ester groups (1.3 per disaccharide unit) (184). The structural similarity of this polysaccharide to other chondroitin sulphates is shown by the degradation with *Proteus vulgaris* chondroitinase to the same unsaturated aldobiouronic acid and its mono- and disulphates. The resistance to attack by periodate of the disulphate suggests that sulphate groups are attached to C₆ of *N*-acetyl-D-galactosamine and C₂ or C₃ of D-glucuronic acid residues.

Earlier investigations, which were reported in a previous review (187), indicated that dermatan sulphate (derman sulphate, chondroitin sulphate B, or β -heparin) differed from chondroitin 4-sulphate only in the stereochemistry at C₆ of the hexuronic acid residue, α -L-idopyranosyluronic acid residues in the former replacing the β -D-glucopyranosyluronic acid residues in the latter. The correctness of this conclusion is supported by the isolation, as common degradation products from the two polysaccharides, of the unsaturated aldobiouronic acid, 3-*O*-(β -D-4,5-glucoseenpyranosyluronic acid)-*N*-acetyl-D-galactosamine (188), and its 4-sulphate (184). The configurational difference between the hexuronic acid residues in the two polysaccharides is eliminated during the formation of these compounds.

Heparin and heparitin sulphate.—The identity of the hexuronic acid component of heparin has been conclusively proved by the isolation of D-glucuronic acid on hydrolysis of heparin *N*-acetate (189) and of D-glucose on hydrolysis of carboxyl-reduced heparin (24, 190). The isolation of D-erythronic acid when *N*-acetylated desulphated heparin is oxidized with periodate, reduced with borohydride, and hydrolyzed, confirms that the glucosamine-glucuronic acid linkage is 1 \rightarrow 4 (191). Half of the hexuronic acid

residues in heparin carry *O*-sulphate groups since they are resistant to attack by periodate (191, 192). The sulphate ester groups have been located at C₃ since deamination of *N*-desulphated heparin affords a mixture of sulphated derivatives of *O*-(*D*-glucopyranosyluronic acid)-2,5-anhydro-*D*-mannose in which all the glucuronic acid units are cleaved by periodate (191). The nature of the *D*-glucuronic acid-*D*-glucosamine linkages in heparin has not been finally established. Danishefsky, Eiber & Carr (193) have isolated an aldobiouronic acid from the partial acid hydrolysis of *N*-acetyl heparin which is claimed to contain a 1→6 linkage on the basis of Morgan-Elson and Elson-Morgan reactions. On the other hand, on the basis of similar reactions of unfractionated oligosaccharides formed from heparin itself and the closely related heparitin sulphate, Cifonelli & Dorfman (190) have suggested that heparin contains mainly 1→6 but some 1→4 linkages, whereas heparitin sulphate contains a larger proportion of 1→4 together with some 1→6 linkages. Since heparitin sulphate is degraded to an α -keto acid (presumably *via* unsaturated acidic oligosaccharides), the *D*-glucosamine-*D*-glucuronic acid linkage is probably 1→4 as in heparin (194).

BLOOD GROUP SUBSTANCES

The chemistry of the blood group specific mucopolysaccharides has been reviewed by Morgan (195). Group A specificity is probably associated with terminal units of *O*-(*N*-acetyl- α -*D*-glucosaminyl)-(1→3)-*D*-galactose, and group B specificity with end groups of *O*- α -*D*-galactosyl-(1→3)-*D*-galactose, whilst H activity is associated with α -*L*-fucosyl end groups. From the examination of a number of oligosaccharides from human milk (196, 196a) it has been suggested that Le^a specificity is associated with the branched trisaccharide unit, bis(3,4-di-*O*- α -*L*-fucopyranosyl)-*N*-acetyl-*D*-glucosamine, whilst Le^b specificity seems to require the attachment of two α -*L*-fucopyranosyl end groups, one to each of two contiguous sugar residues with the *D*-galacto and *D*-gluco configurations (195). The cross-reactivity of degraded blood group substances with pneumococcus Type XIV polysaccharide is probably caused by the exposure of terminal units of *O*- β -*D*-galactopyranosyl-(1→4)-*N*-acetyl-*D*-glucosamine. The blood group H(O) activity shown by certain plant polysaccharides seems to be associated with 2-*O*-methyl-*L*-fucose residues (197). Acid degradation of polysaccharides from *Taxus cuspidata* and *Sassafras albidum* results in release of 2-*O*-methyl-*L*-fucose and oligosaccharides, and is accompanied by loss of blood group activity (198).

The striking changes in the specificity of blood group substances which may be induced by enzymes (especially from *Trichomonas foetus*) have been reviewed by Watkins (199). Degradation of B substances results in release of galactose and affords an H active substance, which may in turn be degraded to an Le^a active substance with release of fucose, whilst further enzymic degradation furnishes a mucopolysaccharide possessing Type XIV pneumococcus activity.

Partial acid hydrolysis of human blood group B substances from ovarian cyst fluid gives a number of oligosaccharides, all of which carry fucosyl

nonreducing end groups (200). Blood group specific mucopolysaccharides from human ovarian-cyst fluids may be fractionated into closely similar mucopolysaccharides of the same general composition, but differing in certain physical and chemical properties (201). An interesting sialomucopolysaccharide with both blood group Le^a specificity and virus-receptor activity has been isolated from a single human ovarian-cyst fluid (202). Removal of *N*-acetylneuraminic acid units results in loss of virus-receptor activity, but not of Le^a specificity, whereas *Trichomonas foetus* enzymes destroy the blood group specificity without decreasing the virus-receptor activity. In contrast to α_1 -acid glycoprotein, this mucopolysaccharide is split into fragments of high molecular weight by papain without the prior removal of sialic acid units.

POLYSACCHARIDES FROM MICRO-ORGANISMS

The value of immunological methods for the structural examination of polysaccharides (13) becomes apparent as further details of the structures of various specific *Pneumococcus* polysaccharides are brought to light. The Type I specific polysaccharide (203) is composed of residues of galactose, fucose, glucosamine, and galacturonic acid. Extracts of pneumococcus SI catalyze the synthesis from uridine diphosphate *D*-galacturonic acid of a serologically active polymer which cross-reacts with the Type I polysaccharide, possibly because of a common polygalacturonic acid backbone (204). The Type III pneumococcal capsular polysaccharide may be synthesized from uridine diphosphate *D*-glucose and uridine diphosphate *D*-glucuronic acid by cell-free extracts of *Diplococcus pneumoniae* Type III (205). *Bacillus palustris* produces two distinct inducible depolymerases which specifically hydrolyze Types III and VIII pneumococcal capsular polysaccharides (206). Although both enzymes attack the glycosiduronic acid linkages of the cellobiouronic acid units in the polysaccharide chains, the S3-depolymerase requires that the disaccharide unit should be preceded by a *D*-glucose residue linked 1,3 β , and the S8-depolymerase that the disaccharide unit is followed by a *D*-glucose unit linked 1,4 β . The Type V specific polysaccharide contains as constituents two epimeric 2-acetamido-2,6-dideoxyhexoses, *N*-acetyl-L-fucosamine (*N*-acetyl-pneumosamine) and *N*-acetyl-6-deoxy-L-talosamine (207), *D*-glucose and *D*-glucuronic acid (208). An important part of the polysaccharide structure consists of repeating units of *O*-(*D*-glucopyranosyluronic acid)-(1 \rightarrow 3)-*N*-acetyl-6-deoxy-L-talosamine, to some of which *D*-glucose residues are attached (208). The Type VI specific polysaccharide shows structural similarities to the teichoic acids in containing glycosyl-ribitol repeating units mutually joined through phosphodiester linkages (209, 209a). The repeating unit, $[-2-O-D-galactopyranosyl-(1\rightarrow3)-O-D-glycopyranosyl-(1\rightarrow3)-O-L-rhamnopyranosyl-(1\rightarrow3)-ribitol-2 \text{ or } 1-PO_4H-]$, has been proposed. In addition to the disaccharides to which the probable structures, 4-*O*- β -*D*-glucopyranosyl-2-acetamido-2-deoxy-*D*-glucose and 3-*O*-(2-acetamido-2-deoxy- β -*D*-glucopyranosyl)-*D*-galactose, were previously assigned (210), partial acid hydrolysis of the Type XIV specific polysaccharide furnishes three

neutral disaccharides, a galactosylglucose (probably lactose), and a galactobiose and a glucosylgalactose, both probably containing 1→3 linkages, and a mixture of trisaccharides, one of which is probably *O*-galactosyl-(1→4)-*O*-glucosyl-(1→4)-*N*-acetylglucosamine (211).

The chemistry and immunochemistry of the O antigenic factors of *Salmonella* polysaccharides have been reviewed (212). From detailed structural studies of the somatic polysaccharide from *Salmonella typhi* using periodate oxidation (213), partial hydrolysis (214), and methylation (215), the nature of the terminal oligosaccharides units responsible for the antigenic specificity of factors 9 and 12 has been established. Artificial antigens with colitose (3-deoxy-L-fucose) as the determinant group have been secured by coupling *p*-aminophenyl α - and β -colitosides with bovalbumin (216). Both α - and β -colitose *p*-phenylazo-bovalbumins possess the immunological properties of the somatic polysaccharides of *Escherichia coli*. The serological specificity of the cell wall carbohydrates of group A streptococci has been shown to be dependent primarily on terminal *N*-acetyl glucosamine units (217). A secondary specificity due to rhamnose units is also present and may be unmasked by enzymic removal of *N*-acetyl glucosamine units. In contrast the serological specificity of group C hemolytic streptococci is due to *N*-acetyl galactosamine units (218).

Like baker's yeast (*Saccharomyces cerevisiae*), the pathogenic yeast *Candida albicans* contains a glucan and a mannan as cell wall constituents (219). The glucan contains 1→3 and 1→6 β -D-glucopyranose residues, but in contrast to the glucan from baker's yeast the polysaccharide is highly branched and contains a high proportion of 1→6 linkages. The highly branched mannan contains 1→2 and 1→6-linked α -D-mannopyranose residues, the detailed sequences of which remain to be established; it differs from other yeast mannans in being more highly branched and in the absence of 1→3 linkages. Although earlier methylation studies indicated that baker's yeast mannan contained 1,2, 1,3, and 1,6 linkages (220, 221), the major products of partial acid hydrolysis are 6-*O*- α -D-mannopyranosyl-D-mannose and the polymer homologous tri- and tetrasaccharides; under milder conditions of hydrolysis a second disaccharide, probably the 1→2-linked isomer, may be isolated, but no oligosaccharides containing 1→3 linkages have been detected (222, 222a). A mannan belonging to the same structural family and containing 1,2, 1,3, and 1,6 linkages is elaborated by *Bacillus polymyxa* (223). The yeast *Cryptococcus laurentii* produces a glucan containing a variety of different linkages and an acidic polysaccharide, which probably contains a backbone of D-mannose residues to which are attached D-xylose and D-glucuronic acid residues (224).

A serologically active polysaccharide from *Citrobacter freundii* contains equimolecular proportions of *N*-acetylneuraminic acid and *N*-acetyl glucosamine residues (225). Galactosamine polymers in which one-third of the residues are *N*-acetylated are obtained from *Aspergillus parasiticus* (226). Teichuronic acid, a mucopolysaccharide which is composed of equimolecular proportions of glucuronic acid and *N*-acetyl galactosamine, has been isolated

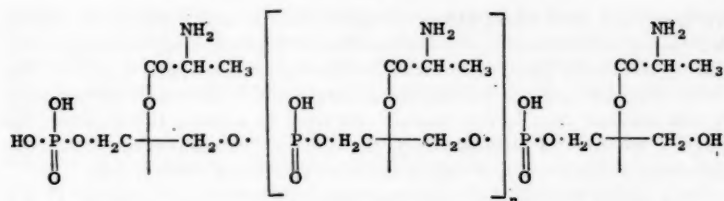
from wall preparations from vegetative cells of *Bacillus subtilis*, where it is associated with a glycopeptide containing glucosamine and muramic acid units (227).

TEICHOIC ACIDS

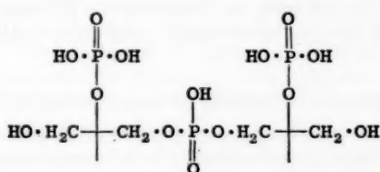
The teichoic acids (228) are polymers of glycerophosphate or ribitol phosphate in which adjacent polyol residues are joined together through phosphodiester linkages. Both types bear D-alanine residues in ester linkage with hydroxyl groups in the polymer, and the latter type also carry glycosidically bound sugar residues. They occur in cells and walls of certain Gram-positive bacteria. Glycerol teichoic acids have been found in both cells and walls, whereas ribitol teichoic acids have been located only in the cell walls (229, 230). Studies of native cell wall structures in a number of organisms indicate that the teichoic acid has free phosphate and amino groups, and is presumably held in the wall through ionic linkages and hydrogen bonds (231). It has been suggested that the ionic nature of teichoic acids in walls is consistent with the view that these polymers may regulate the passage of ions between the organisms and its environment (231).

All samples of glycerol teichoic acid which have been studied are similar in chemical composition in that they are polymers of a unit composed of D-alanine, glycerol, and phosphate (229). The intracellular glycerol teichoic acid from a strain of *Lactobacillus casei* has been examined in detail and the following structure (I) has been advanced (232). Acid hydrolysis of the glycerol teichoic acid gives glycerol, its mono- and diphosphates, inorganic phosphate, and D-alanine, whilst alkaline hydrolysis furnishes as a key degradation product the alkali-stable diglycerol triphosphate (II) whose structure was established by hydrolysis to diglycerol phosphate (III) with phosphomonoesterase (232). The D-alanine residues are in ester linkage with hydroxyl groups in the polymer since they are readily removed with dilute ammonia or hydroxylamine to give alanine amide or hydroxamate.

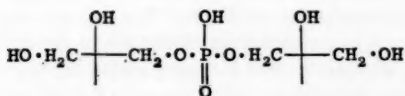
Three ribitol teichoic acids have been examined in detail and they appear to contain common basal chains in which ribitol residues are joined through phosphodiester linkages, D-alanine residues are attached by ester linkages to ribitol, but the polymers differ markedly in the nature and number of sugar residues which are glycosidically linked to ribitol. Structure IV has been proposed for the ribitol teichoic acid from *Bacillus subtilis* walls (233, 234). Alkaline hydrolysis of the polymer gives a mixture of 1- and 2-monophosphates and 1,5- and 2,5-diphosphates of 4-O-(β -D-glucopyranosyl)-D-ribitol. Treatment of the phosphates with prostatic phosphatase furnishes the parent 4-O-(β -D-glucopyranosyl)-D-ribitol whose structure has been established by degradative (233) and synthetic (235) studies. Periodate oxidation of the teichoic acid, before and after removal of D-alanine ester residues, has shown that the glucosylribitol residues are joined through phosphodiester linkages at positions 1 and 5 of ribitol and that the D-alanine residues are linked to C₃ or C₄ of ribitol residues.



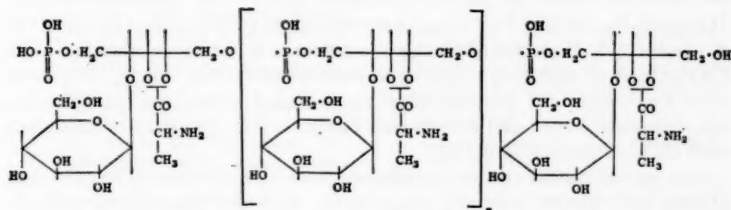
I



II



III



IV

STRUCTURES I-IV

Although similar in composition to the teichoic acid from *B. subtilis* the polymer from the walls of *Lactobacillus arabinosus* differs in that both mono- and diglucoside residues are present and that the glucosidic linkages all have the α -configuration (236). Hydrolysis of this teichoic acid followed

by treatment with phosphatase furnishes, as the major products, ribitol, 4-*O*-(α -D-glucopyranosyl)-D-ribitol, and 3,4-di-*O*-(α -D-glucopyranosyl)-D-ribitol, together with small amounts of 3-*O*-(α -D-glucopyranosyl)-D-ribitol. The results indicate a much less regular arrangement of glucosidic substituents in this polymer than in the teichoic acid from *B. subtilis*; furthermore, the relative amounts of unsubstituted, the different monosubstituted, and disubstituted ribitol residues vary in different samples of teichoic acid.

The ribitol teichoic acid from the walls of *Staphylococcus aureus* H is a polymer of ribitol phosphate containing *N*-acetyl-D-glucosamine and D-alanine residues. Alkaline hydrolysis of the polymer followed by treatment of the products with phosphatase furnishes 4-*O*-(*N*-acetyl-D-glucosaminyl)-D-ribitol (237). Although most of the glycosidic linkages are β , α -linkages have been detected in varying amounts in different teichoic acid preparations.

LINKAGES IN GLYCOPEPTIDES AND GLYCOPROTEINS

The carbohydrate prosthetic group of ovine submaxillary mucin has been shown to be the disaccharide α -D-*N*-acetylneuraminyl-(2 \rightarrow 6)-*N*-acetyl-D-galactosamine (238). Similarly linked prosthetic groups are found in bovine submaxillary mucin (239), but whereas the sialic acid in the ovine form is almost exclusively *N*-acetylneuraminic acid, the sialic acid of the bovine is a mixture of *ON*-diacetylneuraminic acid, *N*-acetyl-*O*-diacetylneuraminic acid, and *N*-glycolylneuraminic acid (240). The majority (*ca.* 82 per cent) of the prosthetic groups in ovine submaxillary mucin are linked to the polypeptide chain by glycosidic-ester linkages and may be released by mild alkaline treatment or by reductive cleavage with lithium borohydride (241). The latter treatment results in a marked decrease of the total dicarboxylic acid units of the mucoprotein, indicating that most of the prosthetic groups are attached to the free carboxyl groups of aspartyl and glutamyl residues. It is probable that the remaining 18 per cent of prosthetic groups are involved in alkali-stable *O*-glycosidic linkages to serine or threonine residues, or both. On the basis of viscometric studies on ovine submaxillary mucin, before and after treatment with neuraminidase, an α -linked helical configuration has been proposed (242), and calculations based on this proposal are consistent with the experimental data (243).

Structural studies on the α_1 -acid glycoprotein (orosomucoid) from human plasma have shown that the trisaccharide unit, *O*-(*N*-acetylneuraminyl)-(2 \rightarrow 3)-*O*-D-galactopyranosyl-(1 \rightarrow 4)-*N*-acetyl-D-glucosamine, terminates the oligosaccharide chains (244). Since acid hydrolysis of the glycopeptide affords a galactosylglucosamine and a dimannosylglucosamine-peptide, it has been suggested that a branched-chain oligosaccharide unit is linked through the amino group of glucosamine to the γ -carboxyl group of a glutamyl residue (245). A structure has been proposed for the oligosaccharide chain in which each of the 3- and 6-positions of glucosamine carry tetrasaccharide units linked through mannose, which contain the sequence *N*-acetylneur-

aminic acid \rightarrow galactose \rightarrow glucosamine \rightarrow mannose. Structural studies have also been reported on haptoglobin (246), an α_1 -acid glycoprotein from pleural fluid (247), fetuin, an α_1 -glycoprotein from foetal calf serum (248), human plasma barium α_2 -glycoproteins (249), ovomucoid (250), and ovalbumin (250, 251).

The carbohydrate moiety of the glycopeptide which comprises the cell walls of *Micrococcus lysodeikticus* contains chains with the repeating unit (1 \rightarrow 4)-O-(N-acetyl-D-glucosaminyl)-(1 \rightarrow 6)-O-(N-acetylmuramyl)-(1 \rightarrow 4). Treatment of the cell wall with lysozyme or a *Streptomyces* enzyme releases the disaccharide, O-(N-acetyl-D-glucosaminyl)-(1 \rightarrow 6)-N-acetylmuramic acid (252, 253), and the polymer-homologous tetrasaccharide (252). The disaccharide, O-(N-acetylmuramyl)-(1 \rightarrow 4)-N-acetyl-D-glucosamine, has also been characterized as a lysozyme degradation product (254). When the cell walls are treated with β -(1 \rightarrow 4)-N-acetyl-D-glucosaminidase, two disaccharide-peptide complexes are liberated (255). The simpler of these fragments contains the pentapeptide unit, alanine \rightarrow alanine \rightarrow glutamic acid \rightarrow glycine \rightarrow lysine, attached through an amide linkage from muramic acid to alanine. The second fragment was characterized as a dimer involving an amide linkage between the carboxyl group of one lysine residue to the ϵ -amino group of the other lysine residue. These results indicate that the oligo- or polysaccharide chains of the glycopeptide are cross-linked by decapeptide units through some of the muramic acid residues.

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CHEMISTRY OF THE LIPIDS^{1,2}

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LITERATURE

The number of reviews covering topics in the field of lipid chemistry and metabolism has increased sharply in the past year, in large measure as the result of the editorial policy of the new *Journal of Lipid Research*. These reviews include *The Inositol Phospholipids* by Hawthorne (1), *Mass Spectrometry in Lipid Research* by Ryhage & Stenhagen (2), *Some New Methods for Separation and Analysis of Fatty Acids and Other Lipids* by Fontell, Holman & Lambertsen (3), *Mechanism of Fatty Acid Synthesis* by Wakil (4), *Structure and Specificity of the Lipid Haptens of Animal Cells* by Rapport (5), *The Sulfolipids* by Goldberg (6), *Molecular Complexes in the Isolation and Characterization of Plasma Lipoproteins* by Cornwell & Kruger (7), *The Metabolism of Adipose Tissue In Vitro* by Vaughan (8), *Lysolecithin* by Robinson (9), *Essential Fatty Acids* by Aaes-Jørgensen (10), and *Factors Influencing the Rates of Long-Chain Fatty Acid Oxidation and Synthesis in Mammalian Systems* by Fritz (11). Several reviews written in 1960 appear in the recently published third volume of *Comparative Biochemistry* edited by Florkin & Mason (12). These are *The Comparative Aspects of Fatty Acid Occurrence and Distribution* by Shorland, *Lipids: Steroid Metabolism* by Grant, *Bile Salts: Structure, Distribution and Possible Biological Significance as a Species Character* by Haslewood, *Distribution of Phospholipids* by Dittmer, *Metabolism of Phospholipids* by Dawson, and *Sterols: Structure and Distribution* by the late W. Bergmann.

The Laboratory of Food Chemistry of the National University of Athens has published a well-arranged bibliographic index (13) edited by D. S. Galanos and covering papers published in the field of lipids in 1959 and 1960 by 20 journals. A "Keyword-in-context" *Index to Neurochemistry* (14) that includes the biochemistry of nervous tissue is available for literature references that were published between January 1960 and July 1961.

METHODS

A useful guide to papers covering separation and analysis of all classes of lipids is compiled by Newman, Radin, & Kritchevsky in the *New Methods* section of the *Journal of Lipid Research* and is indexed separately (15).

¹ The survey of literature pertaining to this review was completed October 13, 1961.

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Adsorption chromatography.—Chromatography on silicic acid columns continues to be the major tool for resolving lipid mixtures, with silicic acid-impregnated paper serving as an excellent method of control. A comprehensive review has been presented by Wren (16). Within recent months, the marketing of apparatus for preparing thin layers of adsorbent on glass plates according to the technique developed by Stahl bids fair to supplant methods using impregnated paper because of greatly enhanced convenience, sensitivity, capacity, and speed of operation. The number of papers published applying this method to lipids is still small (17 to 35), a condition that can be expected to change drastically. The applications include steroids and sterol esters (18, 24, 25, 33), tocopherols (31), glycerides (29, 30), epoxy fatty acids (28), phosphatides (20, 32, 34), and serum lipids (19).

Recent advances in adsorption chromatography have emphasized the separation of glycolipids. Wagner & Hörhammer (36) applied formaldehyde-treated papers to the separation of sphingolipids and phosphatides. Scrignar & Ferrans (37) separated cerebrosides and sulfatides from other brain lipids on silicic acid paper. Mumma & Benson (38) used aminoethyl cellulose paper for plant lipids, including phosphatidyl inositol as well as various galactosyl and glucosyl diglycerides. Long & Staples (39) studied the behavior of cerebrosides and sulfatides on columns of silicic acid and of alumina. With certain alumina preparations, galactolipids can be separated completely from cephalins; and with silicic acid, cerebrosides can be separated from sulfatides. Schwarz *et al.* (40) studied the chromatography of human brain sphingolipids on columns of silicic acid.

Gas-liquid chromatography.—Gas-liquid chromatography has been extended to the determination of dimethylacetals for characterizing aldehydogenic lipids (41, 42) and is rapidly being developed for measurement of steroids (43 to 53) and bile acids (54, 55).

LIPID CHEMISTRY

No comprehensive review of plasmalogens is available, and the growing interest in these compounds suggests that a detailed discussion of the subject should be of value. In this paper, we will review the chemistry and biochemistry of these substances and will then present recent advances in the chemistry of glycolipids, including the inositol phosphatides.

PLASMALOGENS CHEMICAL STRUCTURE

The aldehydogenic linkage.—Isolation of a pure aldehydogenic phosphatide from muscle by Feulgen & Bersin in 1939 (56) and identification of its polar component as glycerylphosphorylethanolamine led to the formulation of its structure as a cyclic acetal of glycerol. This formula was accepted by Thannhauser, Boncoddo & Schmidt (57) for the crystalline ethanolamine plasmalogen isolated from brain in 1951 and subsequently recognized as differing in properties from "native" plasmalogens (58, 59). They correctly

surmised that a lipid chain was cleaved during the alkaline hydrolysis step used in the isolation procedure. In retrospect, there is no evidence to indicate that these crystalline compounds were, in fact, cyclic glyceryl acetals, and the composition and chemical properties of the isolated phosphatides are consistent with the view that in both cases the substances were lyso-derivatives (deacylated products) of native plasmalogens containing the aldehydogenic chain linked to glycerol as an α,β -unsaturated ether. The first clue to the correct structure was provided by hydrogenation studies of brain cephalin by Klenk & Debuch (60) and of beef heart lecithin by Rapport & Alonzo (61) in 1954. Catalytic reduction resulted in the loss of aldehydogenic properties and the formation of a linkage between glycerol and one hydrocarbon chain that was stable to both alkali and acid. Several alternative structures to account for this reaction (hemiacetal, α,β -unsaturated ether, unsymmetric acetal) were suggested by Klenk & Debuch (60), but the first evidence for the α,β -unsaturated ether linkage was provided by Rapport, Lerner, Alonzo & Franzl (62, 63) in studies of a pure crystalline lysoplasmalogen (lysophosphatidyl ethanolamine) isolated from muscle. Their studies showed that this compound, which liberated one molecule of higher fatty aldehyde on hydrolysis, contained just one double bond and that the addition of hydrogen to this bond eliminated aldehydogenic reactions. The double bond had therefore to be located between carbon atoms adjacent to an oxygen function, so that hydration and hydrolysis would produce an aldehyde, while hydrogen addition would produce an ether. Using pure lysophosphatidyl ethanolamine as well as pure lysophosphatidyl choline, Rapport & Franzl (64) obtained independent confirmation by means of a specific, stoichiometric iodination reaction (65), which then permitted the results to be applied to total lipid extracts of tissues as well as to pure compounds (66). Debuch (67) then provided additional evidence by ozonolysis of phosphatide preparations enriched in plasmalogen. These yielded some fatty acids with chain lengths of C_{15} and C_{17} , a result consistent with oxidative cleavage of an α,β -unsaturated ether linkage associated with C_{14} and C_{16} saturated hydrocarbon chains. Concurrently, Blietz (68) showed that acid hydrolysis of similar preparations in tritium oxide (T_2O) led to incorporation of tritium into the fatty aldehydes, a result that was not in accord with the hemiacetal structure which had been adopted (69).

Positions of the residues.—The L- α -structure for plasmalogens was first established by Thannhauser, Boncoddio & Schmidt (70) with their isolation of L- α -glycerylphosphorylethanolamine from crystalline lysophosphatidyl ethanolamine of brain. Evidence was available from partially purified phosphatide preparations containing either ethanolamine or choline (60, 71, 72, 73) that two hydrocarbon chains were present per atom of phosphorus, the second being bound as an alkali-labile ester, thus providing structures for plasmalogens closely analogous to phosphatidyl ethanolamine and phosphatidyl choline. For this reason the nomenclature "phosphatidyl ethanolamine," "phosphatidyl choline," etc. was proposed (73) for native plasmalo-

gen phosphatides, and "lysophosphatidal ethanolamine," etc., for deacylated derivatives (63). This nomenclature minimizes the confusion that arises from the use of less precise terminology, such as "plasmalogen," or "choline plasmalogen," "ethanolamine plasmalogen," etc., to designate either native compounds, lyso-derivatives (74), or other structures that may possibly occur [e.g., molecules with two α,β -unsaturated ether chains or with one saturated ether and one α,β -unsaturated ether chain, and true cyclic glyceryl acetal derivatives for which some evidence has recently been provided (see following paragraphs)].

Studies designed to assign the position of attachment of the ester and unsaturated ether chains to the glycerol residue were all carried out with phosphatide preparations that were mixtures of phosphatidyl and phosphatidal analogues; a pure native plasmalogen was not then available. Initial studies of the relative positions of the chains were misleading. Rapport & Franzl (75) interpreted the similarity in the kinetics of the cobra venom-catalyzed hydrolysis of phosphatidyl choline and of a highly purified beef-heart lecithin (60 per cent phosphatidal choline) to indicate that the ester chain was linked to the α -hydroxyl group of glycerol, in accord with the specificity for α -esters assigned at that time to snake venom phospholipases (76). The specificity of these enzymes is now considered to be restricted to β -esters of phosphatides (77 to 79). Chemical evidence was presented by Ansell & Norman (80) suggesting that the aldehydogenic chain might be present in either α or β position. This evidence was based on the observation, now known to be in error, that under the specified conditions of these investigators, the phosphorus in synthetic α -glycerylphosphorylethanolamine was converted quantitatively into inorganic phosphate. Subsequent studies (81, 82) showed that such conversions from both synthetic glycerylphosphorylethanolamine and that derived from tissue lipids were similar (70 per cent), and therefore a deviation from 100 per cent could not be interpreted to indicate two different types of plasmalogen molecules. The correct position of ester and aldehydogenic chains was first established by Marinetti, Erbland & Stotz (83, 84). Crude phosphatides were catalytically reduced and hydrolyzed with alkali to obtain an alkyl ether of glycerol phosphate, which was then partially purified by chromatography. Prolonged refluxing with acid partially hydrolyzed this material to give a glycerol ether in 35 per cent yield after purification by chromatography and recrystallization; it was identified as α -octadecyl glyceryl ether (batyl alcohol). Similar studies were conducted with crude ethanolamine and choline phosphatide fractions of swine and bovine heart. Debuch (85, 86) confirmed these studies for phosphatidal ethanolamine of brain by hydrogenation of the ethanolamine phosphatide fraction followed by cleavage of the phosphate ester with acetic anhydride according to the method of Bevan *et al.* (87). Saponification of the acetate yielded a mixture of glycerol ethers that were shown to be α -substituted by infrared spectra and periodate-oxidation analysis. In contrast, the results of Gray (88), which were discordant, may have resulted

from the migration of the ester chain during the initial mild acid hydrolysis used to convert the plasmalogen component of ox heart lecithin into a lysolecithin. In Gray's reaction sequence the lysolecithins representative of the plasmalogen component were separated by chromatography and identified by the products formed after oxidation with permanganate. Gray's results must still be explained, since they remain the sole evidence in disharmony with the structural assignment of the aldehydogenic chain to the α -position.

The nature of the aldehydogenic chains.—Further developments in plasmalogen structure have recently centered on variations in the aldehydogenic chains. Gray (41) has studied these chains by gas-liquid chromatography of the dimethylacetals obtained on acid hydrolysis of natural products in methanol according to the method of Klenk & Friedrichs (89). In addition to normal C_{12} , C_{13} , C_{14} , C_{15} , C_{16} , C_{17} , and C_{18} derivatives, a number of branched and unsaturated compounds were obtained. Of some 20 aldehyde components derived from the choline phosphatides of ox spleen, nine could be directly identified with synthetically prepared dimethylacetals. Tentative identification of the other components was made from graphs (grids) in which log relative retention volumes on a polar phase were plotted against log relative retention volumes on a nonpolar phase to give linear relationships between homologues of comparable unsaturation. A similar method of analyzing aldehydogenic-chain components was developed independently by Farquhar (42). In this study, quantification of individual chains by gas-liquid chromatography also utilized dimethylacetals, but qualitative identification was based upon gas-liquid chromatography of long-chain alcohols or their acetylated derivatives obtained after reduction of aldehydes with lithium aluminum hydride. Reference compounds were secured by reduction of fatty acids of known structure. Nineteen different aldehydic chains were detected in human red cell lipids. Schogt, Begemann & Recourt (90) determined the character of the aldehydogenic chains in phosphatide and non-phosphatide plasmalogens of ox heart and milk fat by gas-liquid chromatography of the fatty acid methyl esters obtained after oxidation of the aldehydes with silver oxide. From eight to ten components were detected, with 25 to 35 per cent showing branched chains. Branching in positions α , β , and γ to the terminal carbon was established by determining the ketones formed on oxidation of the fatty acids with chromic acid.

Other aldehydogenic phosphatides.—Plasmalogens with structures other than phosphatidal choline, phosphatidal ethanolamine, and phosphatidal serine have been reported to exist in very low concentrations in a variety of animal tissues. Among the phosphatides, Hübscher & Clark (91) found 15 per cent of plasmalogen in an almost pure phosphatidic acid preparation isolated from rat liver. Smaller amounts in phosphatidic acid preparations from ox and pig liver were within the limits of nitrogen-containing impurities and therefore cannot be attributed to plasmalogenic acid. On paper chromatograms of lipids extracted from infarcted canine myocardium, Hack &

Ferrans (92) have detected a spot staining for both plasmalogen and phosphorus and migrating with a velocity midway between that of phosphatidic acid and phosphatidyl ethanolamine. Hack (93) has also reported a plasmalogen-staining spot migrating with the velocity of phosphatidyl inositol. Several reports indicate the existence of true glyceryl acetal structures. One of the best documented reports is that of Bergmann & Landowne (94) who isolated from a sea anemone, *Anthopleura elegantissima*, a choline phosphatide generating fatty aldehyde and containing 5.30 per cent phosphorus, whose infrared spectrum indicated the absence of both carbonyl and free hydroxyl groups. These properties are best explained by a cyclic acetal structure. However, the substance was also reported to give a "fairly rapid" positive test with Schiff's reagent (fuchsin-sulfurous acid), a property notably inconsistent with the slow reactivity of glyceryl acetals (95). Further study of this interesting natural product would seem to be warranted. Two preliminary reports suggest the formation of cyclic acetals as artifacts. Pietruszko & Gray (96) interpreted a 20 per cent decrease in reactivity with Schiff's reagent, which was noted during alkaline or enzymatic hydrolysis of ox-heart lecithin, as indicating the formation of a glyceryl acetal. A product was isolated that was characterized solely in terms of its slow reactivity with Schiff's reagent. Davenport & Dawson (97) isolated a product after alkaline hydrolysis of ox-heart lipids that was Schiff-positive, stable to acid at 37°C, and gave the same infrared spectrum as synthetic 2'-aminoethyl-*cis*-2:3-0-hexadecylidene-1-glycerophosphate. They found, under similar conditions of alkaline hydrolysis, that lysophosphatidyl ethanolamine was not converted into the cyclic acetal. It is indeed remarkable that glyceryl acetals are not readily formed from lysoplasmalogens, inasmuch as ring closure occurs in model compounds with considerable release of energy (98).

Nonphosphatide plasmalogens.—The presence of nonphosphatide plasmalogens in low concentrations in animal tissues seems to be reasonably certain. Schogt, Begemann & Koster (99) described long-chain aldehydogenic lipids yielding 50, 65, and 1000 μ g of aldehyde per gram of milk fat, beef tallow, and ox-heart fat, respectively. Aldehyde formation was eliminated by prior catalytic hydrogenation, and therefore the aldehydogenic chains were probably bound as α,β -unsaturated ethers. Eichberg, Gilbertson & Karnovsky (100) presented evidence that about 2 per cent of the neutral lipid of sea-star digestive gland is triglyceride containing one of the three hydrocarbon chains bound as unsaturated ether. Such neutral plasmalogens are found in ram testes at a higher concentration than in other mammalian tissues (81).

Pure plasmalogens.—The first isolation of a pure native plasmalogen was achieved by Gottfried & Rapport (101, 102) who obtained phosphatidyl choline by the selective hydrolysis of the phosphatidyl choline component of beef-heart lecithin with *Crotalus atrox* venom. The molecule contained one fatty acyl chain and one aldehydogenic chain bound as α,β -unsaturated

ether. The fatty acid components were almost completely unsaturated (98 per cent), whereas the aldehydogenic chains were almost completely saturated (94 per cent). Structural features were in complete agreement with properties deduced previously from studies of mixtures. The infrared spectrum showed a maximum at 6.05μ that is characteristic of the α,β -unsaturated ether group as a highly unsymmetrical olefin (103), and this absorption band was not appreciably affected by elimination of the fatty-acyl chain carrying most of the unsaturation. The preparation of several pure plasmalogen derivatives retaining the α,β -unsaturated ether linkage have been described: lysophosphatidal ethanolamine by Thannhauser *et al.* (57) and Rapport *et al.* (63); lysophosphatidal choline by Hartree & Mann (104); and an α -alkenyl- β -acyl diglyceride by Kiyasu & Kennedy (105).

DISTRIBUTION

Plasmalogens are lipids characteristic of the animal kingdom, and references to their existence in plants and bacteria are so rare that the validity of such reports remain in doubt. Until very recently, evidence for the presence of aldehydogenic lipids was based almost exclusively on the Schiff reagent (plasmal reaction) employed either as a histochemical or chemical test. It is well-known that lipids may give rise to aldehydes as a result of oxidative changes occurring at unsaturated bonds, so that low concentrations of "plasmalogen" may in some cases represent artifacts. The introduction of newer methods (see following paragraphs) based on *p*-nitrophenylhydrazone formation, specific iodination of α,β -unsaturated ethers, and especially identification, by gas-liquid chromatography, of aldehyde derivatives that are identifiable with those present in native products should eliminate much of the uncertainty.

The quantitative distribution of plasmalogens may be considered from several points of view because of the complexity of their molecular structures. Thus, the fact that they are very largely phosphatides makes the comparison of their concentrations with those of other phosphatides especially informative. Distribution with respect to type of phosphatide is another basis for comparison, and thirdly, the correlation which is now possible between aldehydogenic chains and type of phosphatide should eventually prove to be most useful in relating structure to function. The importance of considering each type of comparison in its own right is readily seen in considering a tissue like muscle. The concentration of plasmalogen in muscle is quite low on a basis of wet weight or dry weight of tissue. However, the plasmalogen content of muscle lipids is high in comparison with that of other tissues. The low plasmalogen concentration in muscle only reflects the low concentration of all lipids in muscle.

Mammalian tissues.—Mammalian organs contain plasmalogens in appreciable quantities, brain having the most and liver the least, no matter what the standard of reference. A recent quantitative study of the organs of

rat, rabbit, and man by Rapport & Lerner (66) showed that the structure of mammalian plasmalogens could be accounted for almost entirely as α,β -unsaturated ethers, since it was possible with total lipid extracts to correlate measurements of long-chain aldehydes liberated as *p*-nitrophenylhydrazones with the uptake of iodine under conditions specific for α,β -unsaturated ethers. Table I shows the concentration of total plasmalogen for five rabbit and five

TABLE I
SOME REPRESENTATIVE CONCENTRATIONS OF PLASMOLOGEN IN RABBIT
AND RAT ORGANS

Tissue	Plasmalogen Concentrations*					
	Mg† per g fresh tissue (66)	μ moles per g dry tissue (66) (106)		μ moles per g lipid (106) (134)		per cent of total phosphatide (66) (134)
Rabbit brain	12.4	79.0	—	—	—	27 —
Rat brain	11.2	69.0	54.0	148	151	24 21
Rabbit heart	5.7	37.0	—	—	—	32 —
Rat heart	2.1	13.0	14.0	82	89	13 9
Rabbit lung	2.7	19.0	—	—	—	14 —
Rat lung	3.1	20.0	18.0	95	77	15 10
Rabbit skeletal muscle	1.4	7.9	—	—	—	22 —
Rat skeletal muscle	1.3	7.3	6.6	38	43	13 12
Rabbit liver	0.58	2.9	—	—	—	2 —
Rat liver	0.88	3.7	3.3	21	24	4 4

* Corrected by increasing literature values by 8 per cent (see analytical methods).

† Assuming a molecular weight of 750.

rat tissues expressed in several different ways. Although agreement among different investigators using identical methods is good qualitatively, the differences in the quantitative values are sufficient to suggest appreciable biological variation among different strains of animals. A significant discrepancy exists with regard to liver. Norton (106) reported a ratio of enol ether to aldehyde of 0.71 for rat liver in contrast to the value 0.90 recorded by Rapport & Lerner (66), and Morrill (107) found a value for this ratio in mouse liver of 0.42. These investigators each used a different modification of the specific iodination method. Whether these values reflect an unusual aldehydogenic lipid component in liver or the failure of some methods for specific iodination of α,β -unsaturated ethers when applied to such low concentrations as those found in liver must still be determined (see section on meth-

ods). The distribution studies cited so far were based on the measurement of aldehydes as their *p*-nitrophenylhydrazones. Other methods continue to find favor with some investigators, despite an inferior degree of reliability. Webster (108) studied plasmalogen distribution in various human nervous tissues and in a number of tissues of the rat (cerebrum, spinal cord, heart, skeletal muscle, liver) using fuchsin-sulfurous acid (Schiff reaction). He reported that in central white matter (corpus callosum, internal capsule), spinal cord, and sciatic nerve, plasmalogen represents 30 to 35 per cent of the total phosphatide, whereas in grey matter (cortex, putamen) this value is about 18 per cent. By fractionation on alumina columns, he showed that very little (2 to 4 per cent) of the plasmalogen in nervous tissue was present as phosphatidal choline and that most was found as phosphatidal ethanolamine. Hen and rat spinal cord contained almost half the phosphatide as plasmalogen. In non-nervous tissues of rat and hen, a higher proportion of the total plasmalogen was represented by phosphatidal choline.

A hydrolytic method in which phosphatide components are determined from the phosphorus content of water-soluble phosphodiester separated by paper chromatography was developed by Dawson and applied to ovine tissues (109). The plasmalogen concentration found in whole brain, with respect to total phosphatide, was 11 per cent phosphatidal ethanolamine, 0.4 per cent phosphatidal serine, and no phosphatidal choline. This total plasmalogen concentration is appreciably lower than any previously reported in fully myelinated mammalian brain, and the percentage of phosphatidal ethanolamine found in the ethanolamine phosphatide fraction was only 39 per cent, which is a figure in sharp disagreement with concentrations found in the brains of other species (66, 107, 108). The proportion of plasmalogen in the phosphatides of other organs (lung, kidney, skeletal muscle) was also quite low, from which it may be inferred that the hydrolytic method which is used does not correctly distinguish phosphatidal from phosphatidyl components. This hydrolytic method was subsequently applied to plasma and to erythrocytes and "ghosts," or both, of six mammalian species (man, pig, horse, cow, sheep, and goat) by Dawson, Hemington & Lindsay (110). Again plasmalogen contents appear to be in error since the total found in human "ghosts" or whole cells was only 8 to 12 per cent of the total phosphatide, whereas Leupold & Büttner (111) found this concentration to be 16 to 17 per cent, and in the most careful studies to date, Farquhar & Oette (112) and Farquhar (113) determined this concentration to be 22 per cent, with 67 per cent of the ethanolamine phosphoglyceride as phosphatidal ethanolamine, 10 per cent of the choline phosphoglyceride as phosphatidal choline, and 8 per cent of the serine phosphoglyceride as phosphatidal serine. If plasmalogens are partially hydrolyzed prematurely in Dawson's method, their glycerylphosphate esters will be attributed to phosphatidyl compounds. Further studies will undoubtedly resolve these difficulties.

In addition to the work of Webster already cited (108) and of Debuch (69), a systematic examination of eight mouse tissues by Morrill (107)

showed the predominance of plasmalogens in ethanolamine rather than choline phosphoglycerides. The difference between the two classes was greatest in mouse brain, followed in order by kidney, testis, lung, pancreas, spleen, heart, and liver. Other mammalian tissues having this same predominance are rat lymphosarcoma (114), human platelets (115), rat heart (116), ox spleen (117), and ram testes (81). On the other hand, more phosphatidal choline than phosphatidal ethanolamine was found in bovine skeletal muscle (81), bovine heart muscle (118, 119), and equine heart muscle (119); and ram spermatozoa were found to contain phosphatidal choline almost exclusively (120, 121).

Thiele, Schröder & Berg (119) reported plasmalogen concentrations, estimated by means of the Schiff reaction, in heart muscle of nine mammalian species (horse, beef, sheep, dog, rabbit, guinea pig, cat, rat, and mouse). The range of concentrations found among these species was large (from 28 per cent of the phosphatide in the horse to 4.4 per cent in the mouse), and an interesting correlation was made between plasmalogen concentration and body surface area for the smaller animals.

Nonmammalian sources.—The first quantitative study of plasmalogens in lower animals was reported by Rapport & Alonzo (122) who showed that lipids derived from 11 species of marine animals (*Arbacia*, *Asterias*, *Busycon*, *Loligo*, *Metridium*, *Mya*, *Mytilus*, *Pecten*, *Thyone*, *Venus*, and *Xiphosura*) contained relatively high concentrations (64 to 207 μ moles/g lipid), that in structure they were almost entirely α,β -unsaturated ethers, and that in every species, phosphatidal ethanolamine and phosphatidal serine, or both, were the major plasmalogen components rather than phosphatidal choline. These studies have recently been extended by Rapport (123) to individual organs of eight species of mollusk (*Busycon*, *Crassostrea*, *Loligo*, *Mya*, *Mytilus*, *Pecten*, *Spisula*, and *Venus*). Although lipids of most organs had high plasmalogen contents, gill lipids were highest, having up to 400 μ moles/g lipid (30 per cent by weight). These studies were prompted in part by the report of Dumont (124) who found that the posterior gills of the Chinese crab, *Eriocheir*, contained 87 per cent of its lipid as phospholipid, and that this was almost entirely phosphatidal choline. The finding of a plasmalogen content representing 15 per cent of its dry weight in an organ with the capacity to absorb salt against a hundredfold concentration gradient (125) led Dumont to suggest that phosphatidal choline played a role in active ion transport. In contrast, in the salt gland of the gull *Larus*, only 22 per cent of the phosphatide was present as plasmalogen, and this was almost entirely phosphatidal ethanolamine (126).

Hack, Gussin & Lowe (127) have recently completed a qualitative study by paper chromatographic methods of the lipids of 46 invertebrates representing 11 phyla. Phosphatidal ethanolamine was found in most species with only a small phosphatidal choline component. Phosphatidal serine was present in mollusks, and to a lesser extent in leeches, hydra, and planaria. A plasmalogenic acid was detected in leeches. Thiele (128) reported changes in

plasmalogens of the snail, *Helix pomatia*, throughout the annual cycle: the concentration rose toward the end of the hibernation period from 9 to a maximum of 13 per cent of the total phosphatide, and then fell in the mating season to a minimum of 4 per cent. Among protozoa, although *Nyctotherus* served as one of the first sources for the histochemical demonstration of plasmalogens (129), these aldehydogenic lipids were found to account for less than two per cent of the phosphatides in *Tetrahymena pyriformis* (130).

The eggs of marine invertebrates (*Arbacia*, *Asterias*, and *Spisula*) have rather high concentrations of plasmalogen (81); in amphibian eggs it is much lower (107); in hen's egg it is very low (131, 132).

CHEMICAL PROPERTIES

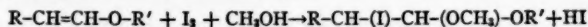
Methods of analysis.—Until 1956, most studies of plasmalogens were based on reactivity with the Schiff reagent using a number of methodological modifications which readily satisfied the demand for exquisite sensitivity. However, this colorimetric reaction is complex and incompletely understood, and despite its great convenience, it no longer serves as a discriminating quantitative tool. There are three reasons for its lack of suitability. First, since the structures of reference compounds differ so much from those of the natural products, adequate standards are not available. Palmityl dimethyl-acetal, the reference compound most frequently used, does not react with the Schiff reagent at the same rate as naturally occurring plasmalogens. Although the extent of reaction is similar under many conditions, the differences in rate occasionally lead to very substantial errors for reasons that are still unknown. The Schiff reaction is thought to occur in two steps, hydrolysis being followed by condensation with the leuco-dye, and differences in reactivity between models and natural products may occur in either step. There is certainly an enormous difference in sensitivity to mercuric ion catalysis in the first step, and the relatively slow reaction of free aldehydes as compared with native plasmalogens (121) suggests a difference in mechanism with regard to the second step, as well. A third reason for the deficiency of the method may be attributed to the essentially aqueous medium in which it is conducted, requiring a number of precautions to eliminate the interferences introduced by (a) the presence of other lipids, and (b) differences in solubility of the reaction products obtained with native plasmalogens having different chemical structures (choline or ethanolamine phosphatides, nonphosphatides). A recent revision in the concentration of plasmalogen in human platelets from 23 per cent to 16 per cent of the total phosphatide (133) was made necessary because of inaccuracies attributed to the Schiff reaction. It had been noted earlier that this reaction may yield results that are too high (134, 158).

The introduction in 1956 of the analytical method based on *p*-nitrophenylhydrazone formation by Wittenberg, Korey & Swenson (134) was a notable advance, since it increased specificity and provided reliable stoichiometry without sacrificing sensitivity. With this method, reference sub-

stances are not required once the value of the molecular extinction of a pure aldehyde *p*-nitrophenylhydrazone is established with a given spectrophotometer, inasmuch as the values for *p*-nitrophenylhydrazones of all aldehydes from C₂ to C₁₈ are identical (135). The increase in specificity resulted from the selection of long chain *p*-nitrophenylhydrazones by partition between hexane and aqueous ethanol, with derivatives below C₁₄ being progressively eliminated with decreasing size. The method is more useful than the Schiff reaction for total plasmalogen since *p*-nitrophenylhydrazone formation with cyclic acetals occurs only a little more slowly than with α,β -unsaturated ethers and does not appear to be appreciably influenced by other lipids. The major difficulty associated with the method results from instability of the chromophore to fluorescent light while in hexane solution. By working in a darkened room, with an indirect, incandescent source of light, and by working rapidly until the hexane solution is diluted with alcohol (see 122), most investigators obtain quite satisfactory results. It is interesting that attempts to adapt the method to the 2,4-dinitrophenylhydrazones, which are much more stable, have met with little success (81), principally because 2,4-dinitrophenylhydrazine forms products during the reaction which give high and variable blank values. The highest analytical yield of aldehyde *p*-nitrophenylhydrazone obtained thus far from pure native and lysoplasmalogens is 92 per cent (64, 102). Values in the literature for plasmalogen concentrations in tissues based on this method have not been corrected to compensate for this inherent inaccuracy and are therefore approximately 8 per cent too low.

One of the most important consequences of establishing the correct structure of native plasmalogens has been the introduction of a new, simple, stoichiometric analytical method utilizing the unique reactivity of the α,β -unsaturated ether bond. The ability of this bond to add iodine under conditions which exclude other types of unsaturation provides a technique for quantification based on a completely independent principle. In contrast to the *p*-nitrophenylhydrazone method, no limitation exists with regard to the length of the hydrocarbon chain bearing the unsaturated ether linkage, and the highest degree of specificity is obtained with respect to aldehydes and their derivatives such as acetals and hemiacetals. Studies with model compounds (64, 136) and pure plasmalogens (64, 102) show that the method suffers from the same degree of inaccuracy as the *p*-nitrophenylhydrazone assay, yielding results that are 7 to 8 per cent low.

The reaction of iodine with an α,β -unsaturated ether linkage is complex, and when it takes place in aqueous methanol, the product is reported to be the unsymmetrical methylacetal (65):



The product is capable of generating higher fatty aldehyde on hydrolysis and would therefore be expected to react rather rapidly with the Schiff reagent. More extensive breakdown may occur, however, and it was found (81) that

lysophosphatidal ethanolamine formed several products under conditons in which the uptake of iodine was quantitative. Until these reactions are defined, evidence based on reactivity with the Schiff reagent of the products formed by iodination cannot support the suggestion that structures other than the α,β -unsaturated ether are present in native plasmalogens (84).

Several solvent systems and iodine concentrations have been recommended for conducting the specific iodination reaction. Siggia & Edsberg (65) used 50 per cent aqueous methanol and 0.05 *N* iodine; Rapport & Franzl (64) used 50 per cent aqueous methanol and 0.005 *N* iodine; Rapport & Lerner (66) used chloroform-methanol (2:1) and 0.005 *N* iodine; Rapport & Alonzo (122) used chloroform-methanol-water (2:5:5) and 0.004 *N* iodine; and Norton (106) used chloroform-methanol-water (2:1:3) and 0.005 *N* iodine. Based on the suggestion of Sloane-Stanley (137), spectrophotometric ultramicro modifications have been developed using iodine at a concentration of 0.0003 *N* in aqueous methanol (102) or chloroform-methanol-water (107). Ultramicro modifications have not yet been tested extensively, and errors resulting from the attempt to introduce such dilute iodine solutions into an indirect method are still not known. Polarity of the solvent plays an important role in determining the rate of this reaction as indicated by Summerbell & Hyde (138).

The molar ratio of α,β -unsaturated ether to aldehyde (specific iodination to *p*-nitrophenylhydrazone formation) introduced by Rapport & Lerner (66) is an important index for characterizing tissue plasmalogens. The closeness of this value to unity in almost all lipid extracts of tissues studied so far indicates that, as a first approximation, almost all plasmalogens are α,β -unsaturated ethers. Deviations from unity may be explained in alternative ways. Values below one may indicate the presence of compounds such as free aldehydes, hemiacetals, acetals, ketones and their derivatives, or other compounds that form *p*-nitrophenylhydrazones but do not react with iodine. Aside from liver (see above) low values of the unsaturated ether to aldehyde ratio were found in extracts of *Arbacia*, *Asterias*, and *Busycon* (122). These low values may be explained by a subsequent report that an addition reaction at the unsaturated ether linkage can sometimes be observed to proceed in chloroform-methanol extracts of marine animal tissues (139). Morrill (107) found low values to be associated most frequently with the neutral lipid fraction eluted from silicic acid. Two additional factors that must be considered in these cases are the possible presence of steroids that form *p*-nitrophenylhydrazones and the possibility that the iodination reaction does not go to completion in the presence of a large excess of neutral lipid.

Values greater than unity for the ratio of specific iodination to *p*-nitrophenylhydrazone formation are obtained with considerable frequency, and Rapport & Lerner (66) recorded 16 instances of values 15 to 20 per cent in excess of one, most of these being found with extracts of human tissues. Gerstl (140) has found even higher values for an extract of human embryonic brain, and Rapport (81) found values 30 to 90 per cent too high with some

extracts of *Metridium* and of fertilized *Arbacia* eggs. Morrill (107) found such high values to be characteristic of the choline phosphatide fraction. A possible explanation for these observations is that some α,β -unsaturated ether structures are associated with chains shorter than C_{12} . Another possibility is that some lipids, or components of lipids extracts that are still unidentified, are capable of adding iodine or being oxidized by iodine under the mild conditions employed. Further studies will be required to clarify this point.

Gas-liquid chromatography of dimethylacetals developed by Gray (41) and Farquhar (42) may be the most powerful tool so far developed for characterizing aldehydogenic lipids both qualitatively and quantitatively. In conjunction with silicic acid chromatography, it is possible to differentiate the individual components in a given lipid class. Thus Gray (117) has shown that the aldehydogenic chains in the phosphatidal ethanolamine plus phosphatidal serine fractions of ox spleen differ markedly from those found in the small phosphatidal choline component, particularly with respect to chains forming a branched, saturated C_{15} aldehyde and the normal, saturated C_{18} aldehyde. Striking differences of a similar nature were also noted in comparing the aldehydogenic chains of these different classes of plasmalogens in ox heart, ox liver, and pig heart, each of which generates from 9 to 17 different aldehydic components. In the phosphatidal choline fraction of ox spleen, half of the aldehydogenic chains were branched.

Since plasmalogens can be converted readily into lysophosphatides by hydrolysis with 90 per cent acetic acid (88), it is possible to determine the fatty acid chains present in the different classes of plasmalogens by separating the lysophosphatides from their diacyl analogues on silicic acid columns and then determining the fatty acids present in the lysophosphatide fraction. Consistent with studies of the unsaturated character of the fatty acid residues in phosphatidal choline of ox heart (72, 75) and in phosphatidal ethanolamine of ox brain (69), Gray found that the fatty acids in the phosphatidal cholines of ox heart and pig heart were about 90 per cent unsaturated. However, in this fraction derived from both ox spleen and ram semen, almost 60 per cent of the acids were saturated, as was also true of the ethanolamine plus serine fraction of ox liver. From 25 to 50 per cent of the fatty acids were saturated in other plasmalogen fractions obtained from ox spleen, liver, and heart. One may question whether these patterns are truly characteristic of different tissues or whether they represent seasonal or nutritional variations. It is of interest, in this connection, to compare Gray's results with the distribution of lipid chains found in the pure phosphatidal choline of ox heart isolated by Gottfried & Rapport (102). For the aldehydogenic chains $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$, the content of the pure plasmalogen was 59, 9, and 21 per cent, respectively, while Gray obtained 68, 1.5, and 9 per cent with his tissue fraction. For the fatty acid chains $C_{18:2}$, $C_{20:4}$, $C_{18:1}$, and $C_{20:3}$, Gottfried & Rapport reported 52, 16, 13, and 9 per cent, respectively, whereas Gray found 42, 2.4, 36, and 2.5 per cent. Thus, in Gray's phosphatidal choline

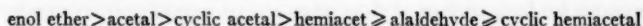
fraction of ox heart in which 90 per cent of the fatty acid residues were unsaturated, the degree of unsaturation was less than that which is usually encountered, and resulted mainly from the replacement of $C_{20:4}$ and $C_{20:5}$ fatty acids by the $C_{18:1}$ acid.

Other chemical reactions.—Unsaturated ethers are known to undergo a number of distinctive chemical reactions (141), including transesterification and addition of carboxyl groups (142, 143, 144), but few of these have been investigated with native plasmalogens. Aside from hydrolysis, hydrogen addition, iodine addition, and ozonolysis already cited in connection with elucidation of structure, only the reaction with mercuric chloride has received any attention. Using a spot test technique on filter paper, Norton (145) found the reaction between phosphatidyl choline and mercuric chloride to be almost instantaneous and the mercuric chloride adduct (hemiacetal) to be labile to acid and mercuric complexing agents. This reaction was used as the basis for a histochemical test (see following paragraphs).

Although it has been suggested that an equilibrium may exist in the presence of acid catalysts between enol ether, acetal, and alcohol (146), there is ample evidence based on stability in alcohol to indicate that enol ethers are not in equilibrium with hemiacetals under ordinary conditions. In contrast, the reaction between free aldehyde and alcohol to form the hemiacetal



does appear to be reversible, as evidenced by the ease of hemiacetal formation and cleavage (147 to 149). The equilibrium strongly favors the hemiacetal when this compound is stabilized by ring formation as in saccharides and 2-hydroxytetrahydropyran (150). Based on chemical reactivity, rates of acid-catalyzed hydrolysis, and reversibility, one may think of several energy levels associated with these related compounds containing a carbon atom in the aldehyde state of oxidation:



From the standpoint of chemical synthesis, α,β -unsaturated ethers are prepared by pyrolysis of acetals or of acylals (148). A recent observation suggests that the energy difference between unsaturated ethers and acetals may be small for models having long hydrocarbon chains. Rapport, Gottfried & Norton (151) unexpectedly observed that the major product formed during the synthesis of octadecyl dimethylacetal was 1-octadecenyl methyl ether. This result has an important bearing on both the chemical synthesis of plasmalogens and on the detection of aldehydogenic chains as dimethylacetals by gas-liquid chromatography. For example, Gray (41) noted the formation of a new compound on chromatographing myristyl dimethylacetal on polyethylene glycol adipate at 180°C which he believed to be the methyl ester of myristic acid from its retention volume. It would now appear more likely that the artifact compound was the 1-tetradecenyl methyl ether.

Ohnishi (152) reported that a compound isolated from rat liver and iden-

tified by qualitative tests as a "choline-containing acetal phosphatide" reacted with thiobarbituric acid to form a red-colored product.

Stability.—The sensitivity of the α,β -unsaturated ether linkage to hydrolysis in the presence of acid catalysts in conjunction with the widespread use of silicic acid chromatography have produced a considerable degree of uncertainty with regard to artifacts. There is little question but that changes in plasmalogens occur unless special precautions are taken, and that considerable disagreement exists among different laboratories on the extent to which such changes occur. Present evidence indicates that native plasmalogens are much more stable than lysoplasmalogens, and that, in the absence of acetic or other acids, prolonged contact with silicic acid is not deleterious if the temperature is kept low. Rapport & Alonzo (81, 122) obtained good recovery of mammalian and marine animal tissue plasmalogens subjected to chromatography at 5°C, and Farquhar (113) found 8°C to be satisfactory for erythrocyte lipids. Hack & Ferrans (153) indicate that even the presence of acetic acid in the developing solvent did not produce noticeable hydrolysis on paper chromatograms in 2 to 5 hours at 2°C. On the other hand, Hartree & Mann (104) reported complete hydrolysis of pure lysophosphatidyl choline in acidic solvents (at ordinary temperature), and Rapport & Lerner (154) found artifacts were obtained from lysophosphatidyl ethanolamine subjected to silicic acid paper chromatography in neutral solvents at room temperature. The data of Kates & James (155) indicate some deterioration of erythrocyte plasmalogens developed on silicic acid paper with solvents containing acetic acid; and the high concentrations of lysophosphatidyl ethanolamine found in erythrocytes of various mammals by de Gier & van Deenen (156) must in large part be attributed to hydrolysis of phosphatidyl ethanolamine. Stability is doubtlessly influenced by the presence of other lipids and by nonlipid impurities, and this may account for nonuniform experiences of different laboratories. Native plasmalogens in crude or highly purified fractions appear to be stable for long periods of time when stored as refrigerated solutions in ethanol or chloroform-methanol (81). Therefore, observations indicating deterioration in short periods of time represent exceptional situations. Rapport (139) observed that some washed chloroform-methanol extracts of marine animal tissues lost their capacity to add iodine over a period of days without any change in their total aldehyde content. These changes, which took place only in extracts that were incompletely washed, were not associated with lowered pH. They were tentatively ascribed to an addition of water or some other substance to the unsaturated ether linkage. Thiele (157, 158, 159) observed the spontaneous formation of free aldehydes on incubating aqueous emulsions of crude phosphatide mixtures at 37°C for several hours. The hydrolytic cleavage apparently is catalyzed by a stable heavy-metal complex. Spontaneous hydrolysis is more pronounced with brain lipids than with lipids from heart or skeletal muscle and is not seen with purified phosphatides. The destructive action of a number of inorganic salts on plasmalogens in aqueous emulsions of purified beef heart phosphatides

was studied by Bergmann & Liebrecht (160). The cations silver, ferrous, and ferric, and the anions bromate and iodide were most destructive.

In studies of bovine adrenal and rat brain tissue, Norton, Gelfand & Brotz (161) found that fixatives containing aldehydes (formalin, acrolein) sharply reduced the quantity of plasmalogen that could be recovered on extraction. They attributed the change to a reaction of the aldehydes with the α,β -unsaturated ether bond. It was found that after a period of fixation sufficient to produce a negative histological reaction with rat brain, at least 35 per cent of the plasmalogen present initially could still be recovered by extraction.

PHYSICAL PROPERTIES

Despite the several reports on isolation of pure plasmalogens and their crystalline derivatives (56, 57, 63, 64, 94, 102, 104, 105), physical characterizations of the substances are scarce. Thus, while Thannhauser *et al.* (57) gave a decomposition point (205°C) for their crystalline lysophosphatid ethanalamine, Feulgen & Bersin (56), Bergmann & Landowne (94), and Rapport *et al.* (63) indicated that the decomposition point was not sharp enough to provide a good physical constant. Thannhauser *et al.* (57) recorded a specific rotation for their compound of $+6.25^\circ$, but Rapport *et al.* (63) found -8.6° for what was presumably the same material. For their "cyclic acetal" of glycerylphosphorylcholine, Bergmann & Landowne (94) gave a specific rotation of -7.85° ; Rapport & Alonzo (162) found -7.6° for their best preparation of crystalline lysophosphatid choline (141). The pure native phosphatid choline of Gottfried & Rapport (102) had a specific rotation of less than 2° . However, an infrared spectrum of this compound showed a characteristic absorption at 6.05μ that may provide a useful physical constant when quantified. Neither Hartree & Mann (104) nor Kiyasu & Kennedy (105) recorded physical measurements of their respective plasmalogen derivatives.

An infrared spectrum presented by Bergmann & Landowne for sea anemone plasmalogen is difficult to interpret because details are lacking on the method used in its determination. The feature that most clearly distinguishes it from related derivatives of glycerylphosphorylcholine is the presence of a double peak in the region of 8 to 8.5μ [the spectrum shown needs to be shifted 0.34μ in the direction of longer wave lengths (163)].

Vinyl ethers have a characteristic, strong infrared absorption band at 8.32μ attributable either to C-O-C deforming in the vinyl ether (136) or to a vinyloxy double bond (164). A second strong band at 6.18μ results from carbon-carbon double bond stretching (136, 164). Neither of these bands is present in plasmalogens. The band at 8.32μ was thought to be masked by overlapping absorption attributable to phosphate (165), but recent experiments of Gottfried & Rapport (102) which successively degraded pure phosphatid choline to the α -1-alkenyl- β -acyl diglyceride and then to the 1-alkenyl monoglyceride revealed that this band was absent. The absorption

band indicating the presence of the α,β -unsaturated ether was found at 6.0 to 6.05 μ and is thus more characteristic of a highly unsymmetrical olefin (103) than of a vinyl group. These data indicate a distinct difference between the properties of the α,β -unsaturated ethers found in nature and vinyl ethers, and it may therefore be anticipated that chemical differences will also be detectable.

It deserves mention that the proximity of the unsaturation to the polar (glyceryl) residue should produce important differences between the properties of monolayers formed by plasmalogens and those obtained with the diacyl analogues (166). Such differences may well play an important role in relations between membrane structure and function.

BIOCHEMICAL PROPERTIES

Few biochemical reactions that are distinctive for plasmalogens are known. Kiyasu & Kennedy (105) showed that the α -alkenyl- β -acyl diglyceride isolated from beef-heart lecithin after cleavage with the lecithinase of *Clostridium perfringens* culture filtrate was similar to D- α,β -diglycerides in accepting phosphorylcholine from cytidine diphosphate choline and phosphorylethanolamine from cytidine diphosphate ethanolamine in the presence of a particulate enzyme from rat liver. Warner & Lands (74) found that the microsomal fraction of rat liver catalyzed the hydrolysis of the α,β -unsaturated ether linkage of lysophosphatidyl choline but not that of the native plasmalogen, phosphatidyl choline. This enzyme may be partly responsible for the loss of endogenous plasmalogen observed in rat liver homogenates incubated at 37°C for several hours by Anderson, Williams & Yarbrow (167). It may be noted that endogenous plasmalogen was found by Norton *et al.* (161) to be undiminished after aerobic incubation for 24 hours at 37°C in beef adrenal homogenates and anaerobically in brain homogenates. Similar stability was seen in muscle homogenates by Thiele *et al.* (119). Gottfried & Rapport (102) showed that rattlesnake venom hydrolyzed the acyl ester bond of pure phosphatidyl choline at a much slower rate than it catalyzed a similar reaction of pure phosphatidyl choline, an observation made earlier by Marinetti, Erbland & Stotz (168) with mixed substrates. In contrast, cobra venom attacked both phosphatidyl and phosphatidyl choline at similar rates (102), confirming an earlier study utilizing mixed substrates under different conditions (75).

Although it is not the function of this review to discuss metabolic aspects, it is perhaps worth noting briefly several reports indicating problems bearing on plasmalogen function that merit additional study. Minder & Abelin (169) found that tissues from adult rats (heart, kidney, muscle, and liver) contained less plasmalogen than tissues from rats three weeks old. An opposite effect was seen in brain. Buddecke & Andresen (170) reported that the plasmalogen content of human aorta decreased with age, in contrast to total lipid, free cholesterol, and esterified cholesterol, all of which increased. In arteriosclerosis a direct relation was noted between the hardness of plaques

and loss of plasmalogen, and this relation was independent of age. Thiele, Schröder & Berg (119) found that in exercised skeletal muscle (rat psoas muscle) the plasmalogen concentration was reduced despite an increase in total phosphatide concentration. These authors also noticed that concentrations of plasmalogen present in heart and skeletal muscle ("red" and "white") of domestic rabbits were higher than those present in the tissues of wild rabbits.

Hartree & Mann (104) observed that lysophosphatidyl choline and lysolecithin had approximately equal activity in lysing horse erythrocytes; however, lysophosphatidyl choline was considerably more effective than lysolecithin in inhibiting the respiration of washed ram spermatozoa.

The subject of plasmalogen changes in serum under the influence of hormones and in various physiological and pathological states has been reviewed by Voit, Seckfort & Busanny-Caspari (171).

HISTOCHEMISTRY

The fact that plasmalogens can be studied by histological methods (plasmal reaction) has stimulated biological interest for many years. These methods introduced a considerable measure of uncertainty because of the sensitivity of the plasmal reaction to artifacts produced during fixation or other manipulation of tissue samples. Very recently, Norton & Korey (172) developed a new histochemical method based on the specific formation of mercuric chloride adducts with α,β -unsaturated ethers in an aqueous medium. Bound mercury is then detected with diphenylcarbazone. In studies based both on extraction and on staining of brain and adrenal tissues after treatment with various fixatives (161, 172) they found that it was possible to distinguish between plasmalogens in two different states of chemical reactivity. Since there is no evidence that the observations are the result of differences in chemical structure of the plasmalogen molecules, differences in reactivity must indicate some difference in interaction between the lipid and other tissue constituents (proteins). It was found (161) that intensity of staining paralleled the concentration of plasmalogen that was labile to fixative, and that a negative plasmal reaction could be obtained with tissue from which 35 to 50 per cent of the plasmalogen initially present could still be extracted. Other evidence consistent with different reactive states of tissue plasmalogens was presented by Terner & Hayes (173). These results serve to emphasize the difficulties associated with reconciling histochemical, biochemical, and chemical data, and the need for parallel studies. As an illustration, the histochemical findings of Seckfort, Busanny-Caspari & Andres (174) may be cited indicating that plasmal staining is not seen in the regenerating rat liver, whereas, after carbon tetrachloride poisoning, plasmal staining is obtained. However, no changes in concentration of liver plasmalogen were found in either condition by Rapport & Alonzo (162). Since it has now been indicated (175) that the initial effect in carbon tetrachloride poisoning is an alteration of endoplasmic reticulum, and since plasmalogens

are concentrated in this fraction (134), the plasmas staining seen in the carbon tetrachloride-poisoned liver may result from the conversion of nonstaining plasmalogen to a reactive form without entailing any change in total concentration.

GLYCOLIPIDS

INOSITOL LIPIDS

The inositol phosphatides have been the subject of considerable structural investigation in the past few years, which has, without doubt, been stimulated by increasing evidence of their role in transport processes and observation of their rapid turnover in brain. Hawthorne (1) has reviewed this chemistry and biochemistry through the early part of 1960. More recent studies have culminated in the characterization of the phosphoinositide complex of brain and proof that monophosphoinositides isolated from various plant and animal sources are identical.

Hawthorne, Kemp & Ellis (176, 177) have shown that liver phosphatidyl inositol yields myo-inositol-1-phosphate on hydrolysis with a rat liver enzyme, and also that the inositol monophosphate obtained from peanuts on alkaline hydrolysis is optically active inositol-1-phosphate. This clarifies Hawthorne's finding (178) that alkaline hydrolysis of liver phosphatidyl inositol yields both inositol-1-phosphate and the optically inactive inositol-2-phosphate. The latter product was evidently produced during hydrolysis via a cyclic intermediate (1). These studies confirm earlier work of Pizer & Ballou on soybean phosphatidyl inositol and show that inositol phosphatides from three sources (liver, soybean, peanut) have the same structure: 1-phosphatidyl-myo-inositol.

The absolute configuration of inositol phosphate from soybean inositide has been determined by Ballou & Pizer (179) to be *L*-myo-inositol-1-phosphate by comparison of the substance derived from the natural product with a synthetic inositol-1-phosphate of known configuration. Brockerhoff (180) has determined the absolute configuration of the phosphatidyl group of beef liver phosphatidyl inositol by studies on the diglyceride obtained with a rat liver enzyme. This phosphatide has the same configuration of the glycerol moiety as all other phosphatides: 1,2-diacyl-*L*-glycerol-3-phosphate. Brockerhoff also found that beef and rat liver inositides, unlike lecithins and triglycerides, do not have positional specificity of saturated and unsaturated fatty acids.

A monophosphoinositide from horse liver has been shown by Brown, Clark, Hall & Letters (181) to have the same structure as that from soybean. Deacylation with hydroxylamine gave 1-(glycerylphosphoryl)-inositol.

Two inositide fractions of beef brain, one extracted with neutral solvents (the "diphosphoinositide" of Folch) and the other extracted as phosphatidopeptide with acidified chloroform-methanol, have been extensively studied. It was found independently by Ellis, Kemp & Hawthorne (182), Grado & Ballou (183, 184), and Dittmer & Dawson (185) that the diphosphoinositide

fraction was a mixture of inositol phosphatides. Hörhammer, Wagner & Holzl (186), in addition to finding evidence of phosphatidyl inositol in brain, also found that "diphosphoinositide" gave three spots when chromatographed on formaldehyde-treated paper. From a neutral solvent extract of beef brain, Dittmer & Dawson (185) isolated a methanol-insoluble lipid containing glycerol, inositol, phosphorus, and ester with molar ratios of 1:1:3:2 (triphosphoinositide A). On alkaline hydrolysis this material gave a product having glycerol, inositol, and phosphate with molar ratios of 1:1:3. They also obtained a monophosphoinositide by column chromatography on alumina and silicic acid. Ellis, Kemp & Hawthorne (182) studied the products obtained by hydrolysis of "diphosphoinositide" with alkali or a liver enzyme, and found evidence for an inositol triphosphate. Grado & Ballou (183, 184) found that alkaline hydrolysis of their preparation yielded a myo-inositol triphosphate (as the major product), an isomeric triphosphate, two inositol diphosphates, and one monophosphate. Structural studies indicated the major product to be inositol-1,4,5(6)-triphosphate. Strickland, Thompson, Subrahmanyam & Rossiter (187), and Thompson, Subrahmanyam & Strickland (188) found that labeled inositide synthesized in the brain was chromatographically similar to 1-phosphatidyl-myo-inositol and gave, on acid hydrolysis, a product electrophoretically identical to inositol-1-phosphate.

Ballou & Tomlinson (189), Tomlinson & Ballou (190), and Brockerhoff & Ballou (191) have now completed the characterization of the four inositol polyphosphates obtained from brain "diphosphoinositide" and have proposed structures for the lipids of this complex. The inositol polyphosphates were characterized as L-myo-inositol-1,4,5-triphosphate (major product), myo-inositol-2,4,5-triphosphate, L-myo-inositol-4,5-diphosphate and myo-inositol-1,4-diphosphate by sequential periodate oxidation, controlled migration of phosphate groups in acid, and partial dephosphorylation by base or phosphoesterase. These differ in a way that suggests migration or elimination of the phosphate group that occupied position 1 in the original lipid (190). Deacylation (191) of the "diphosphoinositide" yielded the following products: 1-(glycerylphosphoryl)-L-myo-inositol, 1-(glycerylphosphoryl)-L-myo-inositol-4-phosphate, and 1-(glycerylphosphoryl)-L-myo-inositol-4,5-diphosphate. Conditions of hydrolysis were such that these products had to be derived from three different lipids; therefore, the various inositol phosphates described earlier were not degradation products of a single triphosphoinositide. The monophosphoinositide of brain is thus shown to have the same polar moiety as monophosphoinositides obtained from other sources. Assuming only the glycerol residue to be acylated, Brockerhoff & Ballou (191) propose the following percentage composition for their particular preparation:

(a) 1-phosphatidyl-L-myo-inositol	20%
(b) 1-phosphatidyl-L-myo-inositol-4 phosphate	22%
(c) 1-phosphatidyl-L-myo-inositol-4,5 diphosphate	58%

This may not represent the relative amounts in whole brain, since some selection is involved in purification.

Ellis & Hawthorne (192) reached similar conclusions. Using mild alkaline hydrolysis and the ion-exchange column techniques of Hübscher, Hawthorne & Kemp (193), they isolated glycerylphosphorylinositol, glycerylphosphorylinositol phosphate, and glycerylphosphorylinositol diphosphate, each of which must derive from a different lipid. Enzymic hydrolysis was helpful in determining the degree of esterification of the phosphate groups.

Dawson & Dittmer (185, 194) isolated a new triphosphoinositide (triphosphoinositide B) from the acid chloroform-methanol-soluble phosphatidopeptide fraction of beef brain. This contains fatty acid, phosphate, glycerol, and inositol in the molar ratios of 6:6:3:2. On mild acid hydrolysis this lipid yielded inositol triphosphate (75 to 80 per cent), some inositol diphosphate, glycerophosphate, glycerol, diglyceride, and fatty acids. With base hydrolysis the main product was glycerylinositol triphosphate. Ellis & Hawthorne (192) believe that their major inositol triphosphate is the same as that Dawson obtained from triphosphoinositide B, indicating the similarity in structure of these two triphosphoinositides isolated from different brain-fractions.

Kemp, Hübscher & Hawthorne (195), in further study of the soluble rat liver enzyme so useful in structural studies of the inositides, found that it hydrolyzes both phosphatidyl inositol (from liver) and impure diphosphoinositide (from beef brain) without attacking other phosphatides. There appear to be two enzymes, one which liberates inositol-1-phosphate and the other, glycerylphosphorylinositol.

Hack (93) described a system for chromatographing the phosphoinositides on silicic acid paper. Hörhammer, Holz & Wagner (196) have continued their chromatographic studies with formaldehyde-treated paper and find that lipids of beef brain give three "diphosphoinositide" spots, whereas two are found with lipids of rat brain, heart, and lung, and only one with lipids of rat kidney.

Klenk & Hendricks (197) have isolated from human brain a new, water-soluble inositol phosphatide complex containing oligosaccharide. It has the following percentage composition: C, 51.05; H, 7.76; N, 2.2; P, 4.7; fatty acids (palmitic, oleic, and stearic), 20; batyl alcohol, 8.4; inositol, 6.1; hexosamine, 5.5; hexoses (mannose, glucose, and galactose), 19.4; ethanolamine, 4.9; and glycerol, 5.4. The mole ratio of inositol to phosphate is 1:4. A tentative structure of the carbohydrate core is offered.

CEREBROSIDES

Dihydrocerasine has been synthesized by Kiss (198). Dihydroceramide, obtained from the reaction of dihydrosphingosine with tetracosanoylchloride, was condensed with tetra-O-acetyl-D-galactopyranosyl bromide to yield a mixture of α - and β -D-galactosides. This mixture was acetylated, isomerized with TiCl_4 , and saponified to give dihydrocerasine, mp 186°C , $[\alpha]_{\text{D}}^{20} +4.63^\circ\text{C}$.

The studies of Shapiro & Flowers (199) have resulted in total syntheses of cerasine and phrenosine. *Erythro*-3-O-benzoyl sphingosine (prepared by ring scission of the previously described phenyl-substituted oxazoline) was resolved via the L- and D-tartrates. The appropriate isomer was acylated directly with the acid chlorides of lignoceric and acetylcerebronic acids. These benzoyl ceramides were condensed with 2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl bromide. Saponification of the acetylated glycosides gave synthetic phrenosine and cerasine, whose physical properties, including infrared spectra, were in good agreement with those of the natural products. The reaction used to form the glycosidic bond and the infrared spectrum of the product confirm the assignment to this bond of the β -configuration. A glucocerebroside was also synthesized and shown to be similar to that isolated from the spleen of a patient having Gaucher's disease.

Radin & Akahori (200, 201), in extending their investigations to human brain cerebroside, found that the saturated normal fatty acids are mostly stearic and lignoceric, with considerable amounts of C_{22} , C_{23} , and C_{25} acids; the saturated α -hydroxy acids are distributed similarly except that the amounts of α -hydroxystearic acid are smaller. The unsaturated acids of both types contained C_{24} acids as the major constituent and also appreciable quantities of C_{25} and C_{26} acids. Sphingosine accounted for 95 per cent of the long chain bases.

The cerebroside from wheat flour was found by Carter and co-workers (202, 203) to contain four long chain bases: phytosphingosine, dihydro-sphingosine, dehydrophytosphingosine, and a new base similar to sphingosine but with the double bond in a different position. Glucose was the only sugar component found, and the major fatty acid was α -hydroxystearic acid.

Carter, Rothfus & Gigg (204) have reported a series of reactions which convert cerebroside to ceramides in excellent over-all yield with retention of the *erythro*-sphingosine configuration. The saccharide ring is opened with periodate, and the resultant dialdehyde is reduced with $NaBH_4$ to a tetraol-acetal, which is then hydrolyzed very easily with dilute acid at room temperature. Ceramides from phrenosine and dihydrocerasine are described. The ceramide from a glucocerebroside (Gaucher spleen) had the "normal" *erythro*-sphingosine configuration.

A cerebroside containing only dihydro-sphingosine was isolated from human brain by Okuhara & Yasuda (205) using solvent fractionation techniques. This material (3.5 g from 90 kg of brain) has a mp of 196–198°C and iodine number of zero. The fatty acid component appears to be solely cerebronic acid, and the hexose is galactose.

Proštenik, Majhofer-Orešćanin, Ries-Lešič, Stanačev & Munk-Weinert (206 to 211) have continued their studies of the sphingolipids, particularly the yeast cerebrins. They find that yeast cerebrin base is 2-amino-1,3,4-trihydroxy-*n*-eicosane (C_{20} -phytosphingosine), and that the anhydro base is 3-amino-2-(*n*-hexadecyl)-4-hydroxytetrahydrofuran (207). Ceramides and some ceramide esters of C_{20} -phytosphingosine and the anhydro base were

prepared containing stearic, palmitic, and α -hydroxy stearic acids (208), as well as the ceramide of the anhydro base containing a cerebronic acid residue (209). Evidence based mostly on the examination of oxidation products of sphingolipid base mixtures (210) indicates the presence of a new sphingolipid base in horse and beef brain. A suggested structure is 2-amino-1,3-dihydroxy-4-eicosene. The *erythro*- and *threo*-C₂₀-dehydrosphingosines (2-amino,1,3-dihydroxyeicosane) have been synthesized and resolved by the following series of reactions. Condensation of nitroethanol with octadecyl aldehyde gave a mixture of the *DL-erythro*- and *DL-threo*-nitro diols. The separated racemates were then reduced catalytically to the bases and resolved as salts of L-glutamic acid (211).

SULFUR-CONTAINING LIPIDS

Little new material on sulfur-containing lipids has appeared since the recent review of Goldberg (6) which covers the chemistry and metabolism of these compounds up to the autumn of 1960.

Hagberg & Svennerholm (212) used a semi-quantitative paper chromatographic method for analysis of sulfatides in their studies of metachromatic leucodystrophy. The size and intensity of the metachromatic spots produced with cresyl violet were compared with those produced by known amounts of pure brain sulfatides.

Bakke & Cornatzer (213, 214) in a study of the metabolism of sulfatides found the concentration of sulfatide sulfur in adult rat tissues to be about 150 $\mu\text{g/g}$ fresh brain and about 9 $\mu\text{g/g}$ fresh liver. These values are similar for organs of man, mouse, and rabbit. Both brain and liver lipids labelled with radioactive sulfur gave two activity peaks on chromatography. Whereas both of the brain lipid peaks were eluted from silicic acid with chloroform-methanol (4:1), one of the liver lipid peaks was eluted with chloroform-methanol (4:1), and the other with chloroform-methanol (1:4).

It is probable that the two brain sulfatides detected by Bakke & Cornatzer correspond to the sulfate esters of cerasine and phrenosine. These esters have been identified by Jatzkewitz (215) in normal human brain and also as the lipids accumulating in metachromatic leucodystrophy. They are separable by chromatography on paper (215) and on thin-layer plates of silicic acid (20). Jatzkewitz (215) has prepared both of these materials by column separation on filter paper (chromatopack) and purification by counter-current distribution. They differed only in the type of fatty acid residues, although the analytical data indicate that the cerasine sulfate preparation is somewhat impure. Sulfation of phrenosine and cerasine with chlorosulfonic acid gave products having chromatographic properties identical to those of the two isolated sulfatides. This sulfation technique may prove to be a useful method of preparing sulfatides, which are purified with difficulty, from the readily available cerebrosides.

Infrared absorption studies of monosaccharide sulfate esters by Lloyd &

Dodgson (216) show that the monosaccharide-6-sulfates, where the sulfate is presumably equatorial with respect to the pyranose ring, are characterized by absorption bands at 1240 cm^{-1} (S=O vibration) and 820 cm^{-1} (C-O-S vibration). In D-glucose-3-sulfate the latter band is shifted to 832 cm^{-1} . Their sample of brain sulfatide had bands at 1240 cm^{-1} and 820 cm^{-1} as did both sulfatides of Jatzkewitz (215), confirming the location of the sulfate ester on the 6-position of the galactopyranose ring. In contrast, the sulfur-containing lipid of green plants of Benson *et al.* was found to have the S=O stretching band, but not the C-O-S band, in accord with its assigned structure as a sulfonic acid (217). This plant lipid, which has been found to occur in all photosynthetic tissues so far investigated, appears to be a sulfonic acid analogue of the major chloroplast lipid, β -D-galactosyl diglyceride. Lepage, Daniel & Benson (217) suggest that the term sulfolipid be restricted to sulfonic acid analogues, and in further characterization of this sulfolipid, have isolated a sulfodeoxyhexopyranosylglycerol from the deacylation products of total alfalfa lipids. The infrared spectrum of the cyclohexylamine salt of this hydrolysis product shows bands at 1035 and 1170 cm^{-1} (S=O stretch) and at 771 and 791 cm^{-1} (C-S stretch in sulfonic acids).

GANGLIOSIDES

The study of gangliosides remains one of the most intriguing areas of lipid research, and in spite of considerable effort in several laboratories, the structural chemistry of this group of compounds is still far from settled. Because of increasing evidence of their role in cerebral physiology, interest in the gangliosides is greater now than at any time since their discovery by Klenk 26 years ago.

It is now generally agreed that the gangliosides, after purification from contaminating phospholipids (and possibly peptides), are a mixture of at least four components. Kuhn, Wiegandt & Egge (218) recently reported the isolation of four crystalline gangliosides; Klenk & Gielen (219, 220, 221) separated two fractions from their preparation, one of which was free of hexosamine and appeared to be composed of two different components, and one of which did contain hexosamine and also produced two spots when chromatographed (219); and Dain, Schmidt & Thannhauser (222) also obtained four fractions by chromatography.

It seems reasonable, therefore, that some of the variations in analyses and yields of hydrolysis products reported by different investigators can be accounted for by probable variations in relative amounts of different ganglioside components obtained with different isolation and purification procedures. For example, Van Heyningen & Miller (223) and Bernheimer & Van Heyningen (224) found that every preparation of gangliosides investigated contained at least three components and that these could be separated into two groups: a fast-moving group (mostly non-metachromatic with cresyl violet) and a slow-moving group (mostly metachromatic). The fast group

contained less sialic acid but the same amount of hexosamine as the slow group. In testing preparations from other laboratories, these investigators found that Klenk's preparation was mostly fast, Chargaff's (225, 226) was mostly slow, and Kuhn's $G_{0.5}$ (called G_1 in reference 227) was slow whereas G_2 (now called G_1 in reference 218) was fast.

Another point that has been clarified is whether gangliosides exist as high molecular weight polymers or whether their molecular weight is the same as their equivalent weight on titration. Klenk & Gielen (220, 221) found for one preparation a molecular weight of about 1500 in dimethylformamide solution and an equivalent weight of 1200 to 1500. Egge (228) for his ganglioside G_2 (now G_1 , ref. 218) found an equivalent weight of 1390 to 1410, and a molecular weight of less than 4000 to 5000 in dimethylformamide. It thus appears that these compounds exist in aqueous solution as associated micelles (221), and as Van Heyningen & Miller (223) point out, the criteria of homogeneity by ultracentrifugation and electrophoresis are therefore not valid indicators of purity. Bernheimer & Van Heyningen (224) also found that amino acids are present in gangliosides extracted from fresh brain but do not accompany gangliosides extracted from acetone-dehydrated brain. This is an interesting finding in view of previous and conflicting reports concerning the peptide moiety.

Kuhn, Wiegandt & Egge (218) designate the four crystalline gangliosides isolated from beef brain as G_1 , G_2 , G_3 , and G_4 , in order of decreasing R_F values on both paper and thin-layer chromatograms. The following table lists the analysis of all components of these fractions in "residues per ganglioside molecule."

	G_1	G_2	G_3	G_4
Fatty Acid	1	1	1	1
Sphingosine	1	1	1	1
Glucose	1	1	1	1
Galactose	2	2	2	2
N-Acetyl galactosamine	1	1	1	1
N-Acetyl neuraminic acid	1	2	2	3

(G_1 is the same as G_2 in ref. 227)

These compositions differ from those reported earlier by Kuhn *et al.* (227), Egge (228), and by Klenk & Gielen (219, 220, 221). Thus the formula suggested by Kuhn *et al.* (227) for G_1 , which provides for only two moles of hexose, must be revised. All four of these gangliosides are related in that G_4 and G_3 are degraded to G_1 , and G_4 is degraded to G_2 , by either acid or receptor-destroying enzyme. Kuhn, Wiegandt, & Egge (218) also state that the same four components are obtained from human brain.

It would be interesting to know if receptor-destroying enzyme (neuraminidase) can further degrade G_1 , since it is known that this enzyme does

not split off more than half of the N-acetyl neuraminic acid of fresh ganglioside (220) [see also Smits (229)]. This question may already have been answered by Bernheimer & Van Heyningen (224) who found that neuraminidase releases more sialic acid from preparations with higher sialic acid content. From Kuhn's fraction G_1 (then called G_2), which is fast-moving and had the lowest sialic acid content of any component, only 2 per cent of the total sialic acid was released on treatment with neuraminidase. Thus it appears that neuraminidase may only liberate the second and third residues of sialic acid in a given ganglioside molecule.

Klenk & Gielen (219) have now separated into two fractions a deacetylated ganglioside preparation from human brain. After reacylation, one (fraction II) was essentially free of hexosamine and had 19.4 per cent neuraminic acid and 33.1 per cent hexose (as galactose); the other (fraction III) had 14.2 per cent galactosamine, 20.6 per cent neuraminic acid, and 38.6 per cent hexose. Fraction II can be hydrolyzed with acid to yield N-acetyl neuraminic acid, galactose, a galactoside-galactose, and a cerebroside containing about equal amounts of glucose and galactose. This fraction must contain at least two different compounds; it is not chromatographically homogeneous. Fraction III yields on acid hydrolysis N-acetyl neuraminic acid, galactose, N-acetyl galactosamine, free galactosamine, a galactosamine disaccharide which may be 3-(2-acetamino-2-desoxy-galactopyranosido)-galactose, and a glucocerebroside. This fraction is also not chromatographically pure. Klenk states that the fast-moving component of fraction III corresponds to Formula 1 previously postulated (221).

Rosenberg & Chargaff (225), and Karkas & Chargaff (226) have studied their preparation of beef brain mucolipid by periodate oxidation and permethylation. This preparation, which has a mole ratio of sialic acid to hexose of 2:3, consumes 1.7 moles of HIO_4 per mole of sialic acid; free sialic acid consumes 2.7 moles of HIO_4 per mole, and the total hydrolysis products after release of all sialic acid consume 3.2 moles of HIO_4 per mole of sialic acid. Since the sialic acid-free gangliosides reduced only 0.5 mole of HIO_4 per mole of sialic acid, they concluded that only half of the sialic acid residues are strictly terminal and that one out of every three hexoses carries two sialic acids (225). However, since this preparation is probably a mixture of different gangliosides, other interpretations are possible. Methylation studies (226) resulted in the identification, after hydrolysis, of 2,3,4,6-tetra-O-methyl galactose, 2,4,6-tri-O-methyl galactose, 2,6-di-O-methyl galactose, and 2,4,6-tri-O-methyl glucose. No structure was proposed to account for these results.

It is interesting to compare these methylation products with those found by Kuhn & Egge (227, 228) and Klenk & Gielen (221). The same tetramethyl galactose and trimethyl galactose were obtained in all three laboratories. Egge (228) considers the tetramethyl galactose to be an artifact resulting from rupture of bonds in the 3-position of the original galactose unit, which can be caused by alkali during permethylation. Both Klenk and Char-

gaff obtained 2,6-dimethyl galactose; Klenk and Kuhn obtained 2,3,6-trimethyl glucose, whereas Chargaff found 2,4,6-trimethyl glucose. Egge considers 2,3,6-trimethyl glucose to be an artifact also. In addition to these components, Kuhn & Egge found 2,6-dimethyl glucose and 3,4,6-trimethyl galactosamine, and Klenk obtained 4,6-dimethyl galactosamine.

Recent studies on the binding of gangliosides, which are dependent on the sialic acid residues, may illuminate the physiological role of these lipids. The excellent review of Smits (229) on free and bound sialic acids covers the chemistry and biology of gangliosides and other sialic acid-containing molecules. Also valuable is the monograph of Gottschalk (230).

In a series of reports, McIlwain (231, 232), Woodman & McIlwain (233), McIlwain & Balakrishnan (234), and Thomson & McIlwain (235) noted that gangliosides restore the excitability to electrical pulses of cerebral tissues which have been kept cold or otherwise inhibited by basic proteins such as protamine. It appears that when tissues are kept cold, histones migrate from the nuclei and block the re-entry of K^+ into cerebral tissue after stimulation. Gangliosides bind to these basic proteins and inhibit their action. The hypothesis is presented that native gangliosides offer acidic sites in the lipid-rich membrane which function in active cation transport and that these sites are occupied preferentially by protamine. Incubation of tissue with protamine leads to a decrease of extractable gangliosides. Wolfe (236) found that gangliosides which have been added to a brain homogenate can be recovered completely, although they are not found in their usual particulate fractions but rather in the nuclear fraction. If protamine is added to a suspension of brain subcellular particles, it completely prevents the extraction of gangliosides.

Van Heyningen & Miller (223) and Van Heyningen (237) confirm that gangliosides fix tetanus toxin very specifically, contrary to the claim of North & Doery (238), and make a careful distinction between fixation and inactivation. Thus ganglioside does not fix diphtheria toxin but does inactivate it, whereas ganglioside fixes tetanus toxin but does not necessarily inactivate it (237). Bernheimer & Van Heyningen (224) found that both tetanus toxin-fixation and influenza virus-inhibiting properties of gangliosides are related to the amount of sialic acid in the preparation.

Harris & Saifer (239, 240) found that metachromasy of ganglioside is suppressed by protamine and that gangliosides bind lysozyme and protamine in a stoichiometric fashion.

From dog erythrocytes, Klenk & Heuer (241) have obtained a ganglioside component that resembles, in all properties, one found in horse erythrocytes. It has equimolar ratios of fatty acid, sphingosine, glucose, galactose, and N-acetylneuraminic acid. The fatty acids are mostly nervonic, lignoceric, and behenic acids. It contains no hexosamine.

Yamakawa, Irie & Iwanaga (242) found that mucolipids of human, sheep, guinea pig, and rabbit erythrocytes contain hexosamine, whereas those of horse and cat have sialic acid instead.

MISCELLANEOUS GLYCOLIPIDS

Rapport (5) has reviewed the subject of animal lipid haptens in an article which summarizes the work of his group on the isolation and chemistry of cytolipin H. This substance is one of a potentially large class of cytosides, a term Rapport has proposed for compounds containing ceramide combined with two monosaccharide residues (243), each member of which should have distinctive immunological reactivity.

Subsequently, Rapport, Graf & Alonzo (243) have shown that human-tumor and ox spleen cytosides, which are similar in composition, react identically with specific antiserum in complement fixation tests. They differ in that the fatty acids of the human tumor lipid are more unsaturated (243, 244). The carbohydrate residues (245) as well as the lipid residues (244) of cytolipin H have been further characterized. Hapten inhibition studies by Rapport, Graf & Yariv (245) show the carbohydrate moiety to be the disaccharide 4-O- β -D-galactopyranosyl-D-glucopyranose (lactose). The fatty acid residues of cytolipin H isolated from both human tumor and ox spleen tissue were found by Rapport, Skipski & Sweeley (244) to consist exclusively of normal fatty acids, with C₂₄ acids predominating and large amounts of C₂₂ and C₁₆ acids. Only small amounts of C₂₀, C₁₈, and C₁₄ acids were found. The long chain bases of a preparation from human tumor consisted mainly of sphingosine (93 per cent); the remainder was a more unsaturated base, possibly dehydrosphingosine. Studies of other lipid haptens of human tissues have been described by Rapport & Graf (246) and Graf, Rapport & Brandt (247).

Carter *et al.* (248) have isolated a number of glycolipids from wheat flour by a combination of solvent fractionation and column chromatography. Among these lipids are a monogalactosyl diglyceride, a digalactosyl diglyceride, and what may be lignoceryl sphingosine trimannoside. The structures of the mono- and digalactosyl glycerol lipids were further investigated by Carter, Hendry & Stanacev (249). Both lipids were shown to be acylated in the 2,3-positions of glycerol. On methylation and hydrolysis the monogalactosyl glycerol compound gave 2,3,4,6-tetra-O-methyl-D-galactose and glycerol; the di-galactosyl glycerol compound gave, in addition, 2,3,4-tri-O-methyl-D-galactose. The complete structures are therefore considered to be 2,3-diacyl-1- β -D-galactopyranosyl-D-glycerol and 2,3-diacyl-1-(α -D-galactopyranosyl-1,6- β -D-galactopyranosyl)-D-glycerol.

A new glycosphingolipid has been isolated from brain (Tay-Sachs disease) by Gatt & Berman (250). This lipid is eluted from silicic acid with 20 per cent methanol in chloroform directly after the cephalin fraction. It was found at a concentration of 1 μ mole/g fresh weight of brain, equally distributed between gray and white matter, and accounted for one-sixth of the total lipid-bound hexosamine in this brain. It was reported to have sphingosine, fatty acid, hexose, and hexosamine in the mole ratios of 1.0:1.0:3.0:0.95 and to be free of sialic acid and phosphate. The authors now state that the hexose to sphingosine ratio is 2 rather than 3 (251).

Nowotny (252, 253) reported a complex phosphomucolipid occurring in endotoxins of *Salmonella* and other Gram-negative bacteria. The hydrolysis products are D-glucosamine (18.1 to 20.5 per cent), fatty acids (50 per cent), phosphate P (1.9 to 2.2 per cent) and amino acids. It appears that about 30 per cent of the fatty acids are bound as amides to the amino groups of glucosamine, and the rest are esterified to hydroxyl groups at the C-3 and C-6 positions. The glucosamine units are linked by phosphodiester groups. No glycerol or sphingosine was present.

Alimova (254) described a complex lipid isolated from diphtheria bacilli. It consists of fatty acids (64 per cent), a very high inorganic residue (9.8 per cent ash), peptide (1.0 per cent total N), and a polysaccharide composed of mannose. The polypeptide contains twelve different amino acids.

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THE CHEMISTRY OF PEPTIDES AND PROTEINS^{1,2}

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Ten years have passed since the pioneer work of Sanger paved the way towards solution of the structure of insulin. Those were the days when the resolution of the structure of proteins of medium molecular weight was considered an unattainable goal. By now there can hardly be anybody who would consider as unrealizable the prediction, made as a joke by Neurath, that at some future time it would be sufficient to state briefly in the experimental section of a paper that "the amino acid sequence has been determined by conventional methods." Each year has witnessed the structural analysis of proteins of higher molecular weight with methods that are increasingly effective. The validity of the proposed structures has been considerably enhanced by independent studies in different laboratories—studies which predominantly yield results that are in excellent agreement. The fact that identical results have been obtained with proteins from different sources, and by different analytical procedures, may stand out as proof that proteins have constant and characteristic compositions. On the other hand, uniquely specific differences in structure may serve as guides for explanation of the fundamental mechanisms of genetics. Another encouraging factor is that proteins of high molecular weight in many cases are made up of monomeric units of a molecular weight that permits investigation by methods so far available.

The accumulation of data on the amino acid sequence of proteins has been paralleled by efforts aimed at the elucidation of intrinsic regularities and of the biological meaning of the sequence. All structures determined up to the first half of 1959 were summarized by Šorm *et al.* (1). Regularities occurring in known primary structures of proteins have been pointed out in the papers of Šorm (2, 3, 4, 5), Gibian (6), and Lanni (7, 8). Morgan (9) has considered sequences from the statistical angle; Yčas (10) and Leslie (11) have dealt with the correlation of sequences to the coding system of nucleic acid and to heredity.

Since the preceding reviews by Perlmann & Diring (12) and Harrap, Gratzer & Doty (13) were devoted for the most part to the problems of the secondary and tertiary structure of proteins and to physico-chemical aspects, this survey will be confined predominantly to the isolation of proteins and

¹ The survey of literature pertaining to this review was completed in August 1961.

² The following abbreviations will be used: CRF (corticotropin releasing factor); DFP (di-isopropyl fluorophosphate); DNFB (dinitrofluorobenzene); DNP (dinitrophenyl); MSH (melanocyte stimulating hormone); NAD (nicotinamide adenine dinucleotide); PTH (phenylthiohydantoin).

TABLE I

ISOLATION OF PROTEINS

Protein	Starting Material	Column Packing or Purification Procedure*	Reference
pepsin	crude pepsin	DEAE	(128)
protease	adrenal glands	DEAE	(138)
bromelain	pineapple	DEAE	(145)
trypsin	bovine pancreas	CMC	(93)
clostripain	<i>Clostridium histolyticum</i>	DEAE	(70, 71)
growth hormone	sheep pituitary	DEAE	(316)
parathormone B	bovine parathyroid	CCD	(201, 202)
prolactin	ovaries	IRC-50	(203)
choriogonadotropin	trophoblastic tumours	DEAE	(317)
bradykinin	bovine serum	CCD, CMC	(205)
lysozyme	T ₂ -phage	CG-50	(252)
phosvitin	egg yolk	DEAE	(263)
ovomucoid	egg white	TEAE	(259)
haemoglobin	horse blood	CMC	(171)
myoglobin	horse muscle	CMC	(181)
pancreatic enzymes	dog pancreas	DEAE	(111)
chymotrypsinogen,			
trypsinogen	pig pancreas	CMC, DEAE	(110)
trypsin	pancreas	CMC	(92)
elastase	pancreatin	CMC	(72)
collagen	muscle	CMC	(318)
gluten	wheat	CMC	(319, 320)
aminoacylase	<i>Brassica campestris</i>	Ca-p	(321)
elaterase	plants	Ca-p, DEAE	(322)
phenolase	broad-bean leaves	DEAE	(323)
amine oxidase	pea seedlings	DEAE	(324)
flavin pyruvate oxidase	<i>Escherichia coli</i>	DEAE	(325)
dehydrogenases	pig liver	DEAE	(326, 327)
β -methylaspartase	<i>Clostridium tetanomorphum</i>	DEAE	(328)
uridine kinase	ascites cells	CMC, DEAE	(329)
phosphomonoesterase		DEAE	(330)
alkaline phosphatase	human organs	CMC, DEAE	(331)
asparagine trans-	rat liver	DEAE	(332)
aminase			
esterases	pig kidney	DEAE	(333)
cytochrome C ₂ and C ₃	microorganisms	CG-50, alumina	(334)
hexokinase	yeast	DEAE	(17)
hydrogenase	<i>Desulphovibrio desulphur.</i>	IRC-50, DEAE	(335)

* Abbreviations used: DEAE, TEAE, CMC for modified celluloses; CCD for countercurrent distribution; and Ca-p for calcium phosphate gel. Various Amberlite resins are designated as CG-50, IRC-50, and XE-64.

TABLE I—(Continued)

Protein	Starting Material	Column Packing or Purification Procedure*	Reference
erythrocyrein	human erythrocytes	DEAE	(336)
β -amylase	wheat	Ca-p	(337)
serum proteins	human serum	DEAE	(15, 16)
haptoglobin	human serum	DEAE	(338, 339)
α -glycoprotein	human serum	CMC	(340)
rennin		DEAE	(341)
thrombin	bovine plasma	DEAE, phosphate cellulose	(342, 343)
meromyosin	rabbit muscle	DEAE	(344)
histones	calf thymus	CMC, IRC-50	(312, 313)
ribonuclease	<i>Bac. subtilis</i>	IRC-50	(292)
ribonuclease	spinach	XE-64, DEAE	(293)
ribonuclease	guinea pig pancreas	IRC-50	(294)
ribonuclease	takadiastase	DEAE	(290)

elucidation of their primary structure. However, due to the vast amount of factual and suggestive data which has accumulated in this field a number of studies, even though of principal importance, have not been quoted here unless they fell within the limits of a wider thematical complex. For the same reason the majority of structural formulas, which require rather a great space, could not be represented. An exception was made with the haemoglobins and myoglobins where the omission of the basic formulas would make the survey incomprehensible.

PREPARATION OF PROTEINS

Of various methods used for the fractionation of proteins those involving chromatographic or electrophoretic procedures will be considered here.

The modern purification technique, of general applicability and of most extensive usage, is chromatography on modified celluloses. Its advantages are high capacity, selectivity in the separation, and preservation of mild "native" conditions. An exhaustive review of different applications up to 1960 has been given by Semenza (14); a number of recent examples are compiled in Table I. Methodological details may be found, e.g., in the paper of Peterson, Wyckoff & Sober (15).

The resolution of 22 different serum proteins by a combination of chromatography and starch block (see below) electrophoresis may serve as a good example of its effectiveness [Jackson *et al.* (16)]. Crystalline hexokinase has been separated on DEAE-cellulose into isozymes of identical specific activity [Trayser & Colowick (17)]. The formation of artifacts by the isolation procedure cannot be suspected in this case since the isozymes also occur in the

parent extract, nor can this phenomenon be accounted for by mutation because identical results were obtained with the extracts of cultures grown from one cell. Most likely, chromatography has distinguished in this case between molecular forms of differing conformation. The successful isolation of a homogeneous peptide of mol wt 6500, from the papain digestion of ovomucoid, provides evidence that modified celluloses can also be applied within the range of lower molecular weights [Morton & Deutsch (18)].

A second technique which has been extensively applied in protein research is gel filtration through a column of particles of modified cross-linked dextran ("Sephadex," Pharmacia, Uppsala, Sweden). This method is based on a chromatographic principle and was originally proposed by Porath & Flodin (19, 20, 21). It provides possibilities of separating substances on the basis of differences in molecular weight. In most cases, therefore, this method can replace not only the desalting of proteins by dialysis but can also be employed for the separation of proteins of different molecular size, for proteins in solutions of low concentration (22), and for ion-exchange in the protein solution. The same principle was utilized by Polson in his chromatographic separation of proteins on granulated agar (23). He succeeded in separating haemocyanin, globulin, haemoglobin, and ribonuclease.

A high fractionating potency can be expected from materials that combine the advantages of gel-filtration with ion-exchange. DEAE-Sephadex, the applicability of which was suggested by Porath (24), may be mentioned by way of example.

The technique of starch-gel electrophoresis described by Poulik & Smithies (373) has been utilized for the fractionation of complex mixtures. The separation of 8 components of human haemoglobin (371), the demonstration of the presence of 16 fractions of basic proteins in ribosomes of pea seedlings (372), the detection of 14 components in rat serum (25), and the fractionation of enzymes of snake venoms combined with specific detections of enzymic activities (26), should be mentioned.

Lastly, two special fractionation techniques should be considered. The first one, used by Julian *et al.* as one step in the purification of glucose-6-phosphate dehydrogenase (27), involves a modification of fractionation by ammonium sulphate: the enzyme, insoluble at 0.40 to 0.65 per cent saturation with ammonium sulphate, is precipitated at 65 per cent saturation in the presence of Celite and the suspension formed is packed into a chromatographic column and subsequently a gradient elution of 65 to 40 per cent saturated ammonium sulphate is applied. In this way a nine-fold enrichment of the enzyme is achieved in one operation.

The isolation of specific antibodies, based on a chromatographic principle, has been described by Brown (28). The antibodies to be fractionated were not held up by ECTEOLA-cellulose in a neutral medium whereas the specific antigen was adsorbed. After the ECTEOLA-cellulose column had been impregnated by this antigen and a mixture of antibodies passed over, only the antibody specific for the adsorbed antigen was retained.

METHODS OF PRIMARY STRUCTURE ESTIMATION

During the past few years the fundamental procedures used for the determination of the amino acid sequence of proteins have achieved a stabilized stage. If the protein contains several chains linked together by disulphide bonds it is usually first cleaved to individual chains, most commonly by reduction and substitution (29), or by S-sulphonation (30). At the same time the protein is thus converted into a form suitable for enzymic digestion. The next step, as a rule, involves a specific proteolytic or chemical cleavage; the fragments formed are separated by ion-exchange chromatography, counter-current distribution, or electrophoresis. The distribution of individual residues in pure peptides is determined by quantitative analysis and the amino acid sequence results from either an additional cleavage to smaller fragments or from stepwise degradation by enzymic or chemical methods. Qualitative analyses of hydrolysates, of amino acids removed by degradation, and separations of small amounts of lower peptides are performed by paper chromatography or high-voltage electrophoresis. Mutual overlappings of fragments from two or more parallel hydrolysates, obtained by proteases of different specificity, then usually provide enough information for the reconstruction of the amino acid sequence of the parent protein. The establishment of complete structures of ribonuclease (124 residues), tobacco mosaic virus (158 residues), and haemoglobin (287 residues in two parallel chains) may serve as telling examples of the high effectiveness of the methods mentioned. The determination of the complete structure of sperm whale myoglobin by Kendrew (31) and Edmundson (32) demonstrated the merits of a combined approach involving x-ray diffraction analysis of high resolving potency and chemical methods.

The methods mentioned have been the subject of critical reviews published recently by Braunitzer (33) and Sanger (34). Therefore, only some of the more important recent trends or observations will be considered here.

Anfinsen & Haber developed a method for the reduction of disulphides in proteins (35). A mixture of the reduced protein and mercaptoethanol in urea solution is chromatographed on Sephadex and can be directly substituted by iodoacetate or *p*-chloromercuribenzoate after emergence from the column. This procedure was tried out by the authors with ribonuclease, chymotrypsinogen, trypsinogen, and lysozyme.

A specific cleavage with N-bromosuccinimide was employed to open the bond tyr.val in hypertensin [Schmir & Cohen (36)]. Treatment of ribonuclease with cyanogen bromide led to a selective cleavage of bonds involving the carboxyl group of methionine, which is converted to homoserine during the reaction [Gross & Witkop (37)]. The specific hydrolysis of aspartic acid peptide bonds in dilute hydrochloric acid was investigated by Grannis (38).

Habeeb (39) studied the N \rightarrow O acyl shift produced by sulphuric acid on lysozyme and globin. The results were unsatisfactory; the reaction was incomplete and, furthermore, the aromatic and sulphur-containing amino acids suffered destruction. Efforts to oxidize proteins by peroxide in the

presence of cupric ions gave even less satisfying results (40). Even though this rather involved reaction produced small fragments of the protein, a great number of amino acids were destroyed and, in the opinion of the authors, the reaction is not likely to be utilized for structural analysis.

Another problem worthy of consideration is the danger of forming chemical artifacts during the treatment of proteins or peptides. Stark *et al.* (41) pointed out that 8 *M* urea solutions, stored for a long period, contain at pH 6 or higher an equilibrium amount of 0.02 *M* cyanate. In such a solution the lysine residues of proteins are converted into homocitrulline. The amount of the artifact can be determined by quantitative analysis. The activation of ribonuclease, observed to occur in 8 *M* urea solutions, is ascribed by the authors to the formation of this artifact. Free cysteine reacts more rapidly than lysine and yields S-carbamyl cysteine, in contrast to protein-bound cysteine which reacts only to a negligible extent. The danger of formation of homocitrulline from the lysine residues can be suppressed by using freshly prepared urea solutions. These findings invite speculation about the possible disturbing role of this reaction in the course of many physico-chemical examinations of protein solutions wherein 8 *M* urea was used.

Niemann (42) has demonstrated the danger of the splitting of amide bonds in peptides during separation by ion-exchange chromatography at elevated temperatures. Ikawa & Snell (43) have shown that during the evaporation of concentrated syrupy solutions in an acidic medium glutamic acid reacts with serine to give O-(γ -glutamyl)-serine. The reaction can be suppressed by limiting the time in which the hydrolysates are maintained in a syrupy stage.

Quantitative analysis of amino acid solutions by ion-exchange chromatography, using automatic recording equipment introduced by Spackman *et al.* (44), has been further developed. An automatic equipment, enabling six parallel analyses and delivering printed results, has been designed by Simmonds & Rowlands (45). Eastoe has reduced the quantity of proteins necessary for analysis to 0.3–0.6 mg and the diameter of the column down to one half (46, 47). Hamilton has carried out the analysis using increased flow rates at high pressure (48). An automatic detection of the effluent amino acids by polarography (49) seems to be less sensitive than the ninhydrin colorimetry frequently used. The chief tendency of future development will apparently lie in shortening the time of analysis by increasing the flow rate and reducing the length of the resin column without reduction in the quality of resolution.

Much recent work has been directed towards development of a quantitative procedure of amino acid analysis by gas chromatography or mass spectrography. Meister *et al.* (50) have converted amino acids into volatile N-acetyl-amino acid n-amyI esters and separated a mixture of 35 components in minimum quantities of amino acid (10^{-10} mole) by gas chromatography. The preparation of the derivatives and the chromatographic resolution

requires two hours. However, the quantitative evaluation involves various difficulties. An effort was made by Zlatkis *et al.* to solve a similar problem by the gas chromatography of aldehydes (51). The possibilities of the application of mass spectrography to both amino acid analysis and the direct estimation of the amino acid sequence of lower peptides have been examined by Biemann (52). The measurement takes 30 to 40 min; the method cannot be used for cysteine. Mass spectroscopy of N-formylaminoacid methyl esters was investigated by Heyns (53).

The advantage of quantitative amino acid analysis by ion-exchange chromatography lies in its accuracy, whereas the relatively long time still required for one run remains as somewhat of a drawback of this method. Gas chromatography or mass spectrography present considerably faster operations; the basic condition to transform amino acids to volatile derivatives necessarily involves the danger of nonquantitative yields of the substitution reactions (36 reactions of 18 components of the mixture take place in a total hydrolysate of protein).

A rapid micro-analysis by thin-layer chromatography has been proposed for the qualitative identification of DNP- and PTH-derivatives of amino acids. The resolution of all DNP-amino acids by two dimensional thin-layer chromatography can be achieved within several hours; the sensitivity of the method amounts to 10^{-3} μ mole of one derivative [Brenner *et al.* (54)]. Qualitative determination of DNP-amino acids by polarography has been described by Vaintraub (55). Di-DNP-histidine can simultaneously be distinguished from the remaining DNP-derivatives. A submicrotechnique permitting the estimation of even 5×10^{-3} μ mole of the PTH-derivative by thin-layer chromatography in silica gel has been developed by Cherbuliez *et al.* (56, 57, 58).

Only two of a number of proposed improvements of paper chromatography and electrophoresis techniques will be mentioned here: the improved system of Hanes for the determination of amino acids (59) and Baglioni's technique of achieving optimum "fingerprints" of peptide mixtures (60)—successful method for studies of pathological haemoglobins.

LOWER UNITS IN THE STRUCTURE OF PROTEINS

Still more and more proteins, originally considered as high-molecular, have been found to consist of subunits. Both haemoglobin and tobacco mosaic virus are formed of chains, each of a molecular weight about 17,000. The monomer of turnip yellow virus has a molecular weight of 21,300 [Harris & Hindley (61)]; potatoXvirus dissociates into 650 units of molecular weight 52,000 each [Reichmann (62)]; γ -globulin dissociates after reduction into chains of one quarter of the original molecular weight [Franěk (63)]; and the molecular weight of the basic chains of β -lactoglobulin is 18,000 [Townsend *et al.* (64)].

Yeast alcohol dehydrogenase has a molecular weight of 151,000. 1,10-

Phenanthroline and 8-hydroxyquinoline-5-sulphonic acid were shown to bind the zinc atom while NAD is removed and the protein component dissociates into four subunits of molecular weight 36,000 [Kägi & Vallee (65)].

Ferritin of molecular weight 500,000, when subjected to specific tryptic hydrolysis, should yield about 600 peptides which is expected in view of the amino acid pattern of the protein. Actually, a very simple mixture containing in several arginine peptides and one peptide of tryptophan is formed. It may thus be assumed that ferritin consists of monomers of a molecular weight of about 16,000 [Saddi *et al.* (66)].

The molecular weight of edestin was originally reported as being 309,000 (67); later the molecular weight of the monomer was found to be 50,000 [Burk & Greenberg (67)]. A chain of molecular weight 16,000 has been isolated from edestin after the reduction of disulphide bonds [Dlouhá *et al.* (68)].

PROTEASES AND PEPTIDASES

The structural investigation of proteins points to several problems that concern enzyme specificity and the conditions of proteolysis. The need of proteases capable of cleaving a protein to a small number of well-defined fragments has become still more obvious. The highest specificity thus far observed has been attributed to trypsin which cleaves its substrate exclusively at bonds involving the carboxyls of lysine and arginine. The preparation of a new protease of the papain type, clostripain, (from *Clostridium histolyticum*) has been described by Labouesse (69, 70, 71); it was found to cleave glucagon at bonds involving the carboxyl of arginine at 300 to 500 times the rate at which the corresponding lysine bonds were split. Other bonds were not hydrolyzed. Should these findings be confirmed with other proteins, clostripain would display the highest specificity so far known.

Pancreatic elastase preferentially attacks bonds involving the carboxyl of neutral aliphatic amino acids [Naughton & Sanger (72)]. Elastase, however, can obviously be separated into two enzymes differing in specificity, as evidenced by the hydrolysis of casein [Lamy *et al.* (73)]. Mandl & Cohen have isolated from *Flavobacterium* an elastase of a higher specificity than that of pancreatic elastase (74).

In addition to proline bonds, leucine aminopeptidase also fails to split the bonds adjacent to phosphorylated serine [Fischer *et al.* (75)]. Only free lysine and glutamine were split off the sequence lys.glu(NH₂).ileu.ser(P).val.arg.

Evidence can be adduced that the specificity of pepsin is not only conditioned by the amino acids involved directly in the bond sensitive to the cleavage [Harris & Roos (76), Ryle (77)]. Thus, e.g., in corticotropin the bond glu./-his.phe is attacked whereas the bond glu.his./-phe is attacked in MSH. Similarly, of two identical sequences, val.ala.cys of ribonuclease, only one is hydrolyzed. Apparently a distinct difference exists between the

specificity of pepsin and that of the parapepsins since the bond glu-/his.phe is hydrolyzed by pepsin free of the parapepsins (77).

An entirely new approach to the problems of proteolysis is outlined in the studies of Katchalski *et al.* (78, 79), who were successful in obtaining new protein derivatives (water-insoluble and enzymatically active) by the coupling of polytyrosylated protease with a copolymer of *p*-amino-DL-phenylalanine. Since the immobilization of the enzyme molecules prevents autolysis, no losses in activity occur in the course of the cleavage. The insoluble enzyme preparation, when diluted with an inert carrier, can serve as a chromatographic column. In two weeks, 4 g of L-arginine methyl ester were cleaved by being passed through a column containing 20 mg of the insoluble trypsin preparation, without any observable drop in its activity (78). Using the same arrangement, myosin, protamine, and insulin were degraded to peptides. An insoluble papain preparation has been employed in structure studies of antiovalbumin (79).

The coupling of proteases with modified cellulose has been described by Mitz & Summari (80). Carboxymethyl cellulose azide when coupled with the protease yielded a derivative of identical or higher activity as compared with that of the parent enzyme. Trypsin and chymotrypsin modified in this manner display a higher activity towards synthetic substrates than to proteins. The carboxymethyl cellulose derivative of trypsin can be used for the activation of chymotrypsinogen.

The course of proteolysis in general has recently been examined by Schlamowitz *et al.* (81). Based on the course of peptic cleavage of bovine serum albumin, trypsin, and ribonuclease they voiced the opinion that proteolysis is a multistage reaction involving the production of fragments of molecular weight ranging from the highest to the lowest figures, and that it is not an "all or none" process in which either the intact protein or small fragments would exist.

The influence of denaturation on proteolysis has been investigated with serum albumin, ribonuclease, ovalbumin, and casein (82, 83). Prellwitz *et al.* (82) have measured the Michaelis constant after denaturation, White, Rupley & Scheraga (83) have examined the influence of temperature on the susceptibility of ribonuclease towards chymotryptic and tryptic hydrolysis at pH 6.5. The greatest difference in the rates of hydrolysis was observed over the region between 40.3° and 43.6° where, obviously, an unfolding of the α -helix occurs.

The nature and concentration of ions in the medium are essential influences in proteolysis. In relation to the concentration of NaCl, the optimum pH for the action of pepsin ranges either between 1.5 and 3 (at $\Gamma/2=0.15$) or there are two different optima of activity, at pH 2 and pH 4 (at $\Gamma/2=0.5$) [Schlamowitz & Peterson (84)]. The course of proteolysis is naturally also affected by the nature of the substrate.

The conclusions which can be drawn from the study of Yon (85) are of

special interest. Monovalent cations (Na^+ , K^+ , NH_4^+) stabilize the native protein towards proteolysis. Chymotrypsin and trypsin are 50 per cent inhibited at $1.5 \times 10^{-2} M$ concentration of ammonium ions, and complete inhibition occurs at concentrations exceeding $0.2 M$. Na^+ or K^+ ($0.36 M$) inhibit these enzymes by 50 per cent. Bivalent metal ions (Ca^{++} , Mg^{++}) form bonds with the substrate and modify its susceptibility towards cleavage; they do not, however, cause inhibition. The effect of copper has a characteristic feature: trypsin is activated provided the substrate contains lysine bonds which can be cleaved, and, on the contrary, inhibited if only arginine bonds are available. Copper has no effect on chymotrypsin under the same conditions. The characteristics of the phenomenon are completely different with denaturated substrates where the susceptibility towards cleavage is increased in the presence of Na^+ . Hummel has demonstrated that the effect of quaternary ammonium ions upon the activity of trypsin is influenced to a considerable degree by the nature of the substituent (86). Excess of protein can inhibit the hydrolysis of synthetic substrates by chymotrypsin; this inhibition is prevented by the addition of bivalent cations and anions [Hofstee (87)]. This finding might somehow be related to the observation of Veremjenko (88) pertaining to the specific inhibition of trypsin by plasma. The inhibition of proteolysis by detergents depends on the nature of the substrate. The digestion of bovine plasma albumin by trypsin or chymotrypsin is inhibited by detergents, while the digestion of α globulin is nearly unaffected [Epstein & Possick (89)].

A high-molecular residue which is not cleaved further, the so-called "core," has been observed in the digests of many proteins. The fact that this "core," after isolation from the mixture, is not digested further by the original enzyme can be explained by either the inaccessibility of further susceptible bonds for the enzymes (sterically unavailable) or by the assumption that the "core" does not contain other bonds to be split. If, however, the "core" is digested by the same enzyme after removal of low-molecular products by dialysis or gel filtration, an inhibition of the enzyme by low-molecular products has to be suspected [Keil (90)].

Microbial proteases.—The subtilisins of various origin have been compared by Hunt & Ottesen (91). The original subtilisin, and the bacterial proteinases Novo and Nagese (Jap.) differ considerably both in specificity and in the "fingerprint" pattern of their tryptic hydrolysates.

Clostripain whose specificity has been mentioned above can be obtained by a relatively simple operation. Approximately 1 g of pure substance may be obtained from 20 liters of the cultivating medium [Labouesse & Gros (79, 71)]. The molecular weight of clostripain is about 90,000.

Chymotrypsin and trypsin.—The investigation of pancreatic proteases has been focused predominantly on zymogens and enzymes of bovine pancreas, because of the simple preparative procedures. Whereas homogeneous preparations of chymotrypsinogen may be obtained by repeated crystallization and chromatography, the purity of trypsinogen and trypsin has remained

the subject of further examination (92, 93, 94). The molecular weight of trypsinogen, as determined by light-scattering and sedimentation analysis, is 24,500 (95). Only four of the nine to ten tyrosine residues of native trypsinogen are ionizable. Complete ionization of all tyrosines occurs above pH 10 where the molecule is unfolded because of denaturation (94).

The conversion of chymotrypsinogen into π -chymotrypsin, studied by rotatory dispersion, is an activation in which an α -helical fraction of 5 to 6 turns is formed [Imahori *et al.* (96)]. The conversion of trypsinogen to trypsin has been effected by the action of liver or spleen mitochondria [Zinnari (97)] or by *Penicillium* kinase [Hofmann (98)]. In the latter case the course of the kinetics was identical with that of the autocatalytic activation and with that of activation by *Aspergillus oryzae* protease. The product of activation was inhomogeneous when examined by end-group analysis.

During the past three years, following upon the review of Hill, Kimmel & Smith (99), the primary structure of chymotrypsinogen has been the subject of exhaustive investigation mainly by Šorm, Keil *et al.* (100, 101), and by Hartley (102, 103). Oxidized chymotrypsinogen or the S-sulpho derivative was used as the starting material. The results so far obtained have been summarized by Keil *et al.* (90, 104) and by Hartley (102). A mutual correlation of these results indicates that the number of residues in the portions thus far resolved approaches the analytical data for chymotrypsinogen. The disulphide bonds have been partly characterized.

A detailed examination of the primary structure of trypsin has been performed mainly by Neurath *et al.* (105) and Šorm *et al.* (106). Similarly, in this case, the sequential analysis has proceeded to an advanced stage. It may be hoped that the elucidation of the structures of these two chief pancreatic proteases will be completed in the near future, thus providing a basis for the investigation of genetic and inter-species differences as has been the case with the haemoglobins. Recently, two fragments, lys.leu.ser.thr.ala.ala.ser and lys.leu.leu.ser.thr.ala.ala.ser, were isolated from a partial hydrolysate of chymotrypsinogen (104). According to the general scheme of the molecule, both sequences originate from the same site. This observation tends to indicate that the principle of small differences in amino acid sequence due to genetic differences is a general feature of proteins.

Unlike the work on bovine chymotrypsinogen A, no essential progress in resolving the primary structure of chymotrypsinogen B has been achieved except for a new isolation procedure [Roverly *et al.* (107)], amino acid analysis [Roverly *et al.* (108), Kassel & Laskowski (109)] and determination of N-terminal half-cystine (108, 110).

The investigation of pancreatic proteases has been extended to other species by Desnuelle *et al.* They followed the overall distribution of enzymes in the pancreatic juice of pigs and dogs (111) by chromatography of the mixtures on DEAE-cellulose. Pure porcine trypsinogen and chymotrypsinogen A have been obtained by Roverly, who determined their amino acid composition and terminal peptides (108, 110, 112). The N-terminus of chy-

motrypsinogen A, both bovine and porcine, is occupied by a half-cystine residue. There exists, however, a considerable difference between the N-terminal residues of porcine and bovine trypsinogen. Thus for bovine trypsinogen one finds H.val.asp⁴.lys, while for porcine trypsinogen we have H.phe.(thr, pro, asp).lys . . . ileu.ala.asp(NH₂)³.OH. A method of purification, and the enzymic properties of trypsin from cattle, sheep, and pig have been described by Vithayathil *et al.* (92). All these proteins gave two components when chromatographed on carboxymethyl cellulose. Trypsin of pig differs distinctly from trypsin of cattle and sheep.

A relationship between structure and function has been sought in further examinations of active centres and modified enzymes. A model of the active centre of proteases has been discussed by Jakovlev & Rozenart (113) and a comparison of the active centres of chymotrypsinogen, trypsin, and elastase has been conducted by Naughton *et al.* (114). The influence of substitution and modification upon activity has been investigated by different approaches. The chief reason for the rather difficult interpretation of some of the results seems to lie in the unspecificity of substitution. Mostly mixtures of compounds substituted to a different degree and at different groups are formed. This is, e.g., the case of acylation reactions. Thus, according to Ciperovic & Losjeva (115), trypsin retained 71 to 75 per cent of activity and chymotrypsin 83 to 85 per cent activity after acetylation of amino groups. Acetylation of 85 to 89 per cent of the groups, however, brought about inactivation [Wong & Liener (116)]. Whereas the acylation of trypsin with lower anhydrides results in inactivation, partial activity is retained when trypsin is treated with higher anhydrides (succinyl-, citraconyl-) (117, 118). A fragment of molecular weight 5500 to 6500, capable of activation and with phenylalanine as the N-terminal residue, was obtained by Viswanatha & Liener (119) from a peptic digest of acetylated trypsinogen. The specificity of this product was broader than that of trypsin. The esterification of chymotrypsinogen with diazoacetamide is not quantitative [Doscher & Willcox (120)]. The resulting mixture of substituted products can be separated on carboxymethyl cellulose, and some of the fractions can be activated.

Trypsin yields with S-CH₃-glucosylisothiourea, which reacts preferentially with histidine, an active crystalline derivative with three histidine residues and three ω -amino groups of lysine substituted [Maekawa & Liener (121, 122)]. This finding, together with isolation of the active fragment from acetyl trypsin (119) which does not contain histidine, provides evidence that histidine is not essential for the activity of trypsin.

The oxidation of one tryptophan in trypsin with N-bromosuccinimide has no influence on the activity of the enzyme [Viswanatha *et al.* (123)]. When chymotrypsin is treated with the same oxidation procedure one tyrosine residue is attacked in addition to the tryptophans. The inactive

³ Or glu (NH₂).

product obtained is able to react with DFP [Viswanatha & Lawson (124)]. The kinetics of the reactions of chymotrypsinogen with DFP and DNFB have been studied exhaustively by Ooms (125) and Ivanov (126) respectively.

Recent studies on the oxidation of chymotrypsinogen and trypsin using sedimentation analysis have confirmed the previous findings that chymotrypsin is split by performic acid to at least three fragments whereas the molecular weight of chymotrypsinogen remains unchanged under identical conditions [Šponar (127)].

Other animal proteases.—Pepsin has been purified by chromatography on DEAE-cellulose [Orechovich *et al.* (128, 129)]. A fragment of pepsin of molecular weight below 10,000 (sedimentation constant $0.56 \times 10^{-3}S$), with one third of the original activity, has been isolated by Tokuyasu & Funatsu by chromatography on Amberlite IRC-50 and Dowex 50×2 (131). The amino acid sequence of the N-terminal portion of pepsinogen, involving eighteen residues, has been established by Loksina & Orechovich (130).

Wallee has studied the active site of carboxypeptidase A (132, 133). Apparently, cysteine and either imidazole or an α -amino group are responsible for the binding of zinc to the enzyme molecule. The amino acid sequence of both ends of the polypeptide chain of carboxypeptidase B has been established by Folk *et al.* as follows: H. thr. ser . . . (val, ser) . asp(NH₂) . thr. OH (134).

Several new preparation procedures for animal proteinases have been described. Proteases of the trypsin type, which are inhibited by soya bean trypsin-inhibitor, have been isolated from the gut of the fish *Etrophus suratensis* [Sundaram & Sarma (135)] and from the larvae of the blow fly *Phormia regina* [Brookes (136)]. Cathepsins, of pH optimum 3 to 4, were obtained from rabbit spleen [Uriel *et al.* (137)], adrenal glands [Todd & Trikojus (138)], and Ehrlich ascites cells [Keilová *et al.* (139)]. Collagen mucoproteinase has been isolated from the pancreas; it occurs together with elastase and splits off from the connective tissue a mucoprotein containing collagen as its main protein component—the so called mucoid₂ [Banga *et al.* (140)].

Plant proteases.—The progress in the work of Smith *et al.* (141, 142, 143) on papain indicates that the solution of the complete structure has approached the final stage. Eleven fragments which account for 168 residues of the molecule of papain have been obtained and the sequence along the middle portion comprising 57 residues has been solved in full. Soejima & Shimura (144) have described an activation of papain, in the absence of ions, using reduction by *p*-thiocresol.

Bromelain, a proteinase from pineapple similar to papain, has been purified on DEAE-cellulose [Ota & Hirohata (145)]. Another plant protease, hurain (from *Hura crepitans*, Euphorbiaceae), is not of the papain type; it is inhibited by trypsin inhibitors [Jaffé & Seidl (146)]. The proteases from *Trifolium repens* [Gainor & Crisley (147)] and from plant tumours [Mosolov & Skarlat (148)] have as yet been less characterized.

TABLE II

AMINO ACID SEQUENCES OF HAEMOGLOBIN CHAINS (α , β , γ) AND MYOGLOBIN (M)

α	β	M	γ	α	β	M	γ
val	val		gly	pro	glu	asp	
	his		his	45 his	ser	arg	
leu	leu		phe	phe	45 phe	phe	
ser	thr	val	thr		gly	45 lys	
pro	5 pro	ala	glu	asp	asp	his	
5 ala	glu	gly	glu	leu	leu	leu	
asp	glu	glu	asp	ser	ser	lys	
lys	lys	5 try	lys	50 his	50 thr	thr	
thr	ser	ser	ala		pro	50 glu	
asp	10 ala	glu	thr		asp	ala	
10 val	val	ileu	ileu		ala	glu	
lys	thr	leu	thr		val	met	
ala	ala	10 lys	ser		55 met	lys	
ala	leu	?	ileu	gly	gly	55 ala	gly
try	15 try	try	try	ser	asp	ser	asp (NH ₂)
15 gly	gly	?	gly	ala	pro	glu	pro
lys	lys	leu	lys	glu	lys	asp	lys
val	val	15 leu	val	55 val	60 val	leu	val
gly	asp	glu	asp (NH ₂)	lys	lys	60 lys	lys
ala		?		gly	ala	val	ala
20 his		leu		his	his	his	his
ala	20 val	val	val	gly	gly	gly	gly
gly	asp	20 ala	glu	60 lys	65 lys	ileu	lys
glu	glu	gly	asp	lys	lys	65 glu	lys
tyr	val	his	ala	val	val	val	val
25 gly	gly	gly	gly	ala	leu	asp	leu
ala	25 gly	lys	gly	asp	gly	his	thr
glu	glu	25 leu	glu	65 ala	70 ala	ala	ser
ala	ala	thr	thr	leu	phe	70 leu	leu
leu	leu	leu	leu	thr	ser	gly	gly
30 glu	gly	ileu	gly	asp	asp	ala	asp
arg	30 arg	ser	arg	ala	gly	ileu	ala
met	leu	30 leu	leu	70 val	75 leu	asp	ileu
phe	leu	phe	leu	ala	ala	75 arg	
leu	val	lys	val	his	his	lys	
35 ser	val	ser	val	val	leu	lys	
phe	35 tyr	his	tyr	asp	asp	gly	
pro	pro	35 pro	pro	75 asp	80 asp	leu	
thr	try	glu	try	met	leu	80 his	
thr	thr	thr	?	pro	lys	glu	
40 lys	glu	leu	?	asp	gly	leu	
thr	40 arg	glu	arg	ala	thr	glu	
tyr	phe	40 lys		80 leu	85 phe	glu	
phe	phe	phe		ser	ala	85 ala	

TABLE II—(Continued)

α	β	M	γ	α	β	M	γ
ala	thr	pro		115 ala	120 lys	asp	
leu	leu	thr		glu	glu	120 glu	
ser	ser	ala		phe	phe	phe	
85 asp	90 glu	his		thr	thr	gly	
leu	leu	90 ser		pro	pro	ala	
his	his	his		120 ala	125 pro	pro	
ala	cys	ala		val	val	125 asp	
his	asp			his	glu	ala	
90 lys	95 lys	lys		ala	ala	gly	
leu	leu	leu		ser	ala	ala	
arg	his	95 phe		125 leu	130 tyr	met	
val	val	lys		asp	glu	130 gly	
asp	asp	ileu		lys	lys	lys	
95 pro	100 pro	pro		phe	val	ala	met
val	glu	ileu		leu	val	leu	val
asp	asp	100 lys		130 ala	135 ala	glu	thr
phe	phe	tyr		ser	gly	135 leu	gly
lys	arg	?		val	val	phe	val
100 leu	105 leu	glu		ser	ala	arg	
leu	leu	his		thr	asp	lys	
ser	gly	105 leu		135 val	140 ala	asp	
his	asp	ser		leu	leu	140 ileu	
cys	val	?		thr	ala	ala	
105 leu	110 leu	ala		ser	his	ala	
leu	val	val		lys	lys	lys	arg
val	cys	110 ileu		140 tyr	145 tyr	tyr	tyr
thr	val	his		arg	his	145 lys	his
leu	leu	val				glu	
110 ala	115 ala	arg				leu	
ala	his	ala				gly	
his	his	115 thr				tyr	
leu	phe	lys				150 gly	
pro	gly	his				glu	
		asp					

HAEMOGLOBIN AND MYOGLOBIN

The solution of the complete structure of human haemoglobin and sperm whale myoglobin stands out as a brilliant success of recent protein structure research.

The structure of the α and β chains of human haemoglobin has been determined exclusively by chemical methods, mainly by the group of Braunitzer (149 to 153, 353 to 355), Hill & Konigsberg (154, 155), and Schroeder (156). The structure of sperm whale myoglobin has been elucidated by a combined approach involving x-ray diffraction analysis [Kendrew

TABLE III
PATHOLOGICAL SUBSTITUTIONS IN HUMAN HAEMOGLOBIN

Haemoglobin	Amino Acid	Residue Number	Reference
I	asp	α 16	(356, 357)
Norfolk	asp	α 57	(358)
M-Boston	tyr	α 58	(158)
M-Emory	tyr	β 63	
M-Milwaukee	glu	β 67	
G-Philadelphia,X	lys	α 68	(359, 360)
G-San José	gly	β 7	(363, 365)
C,X	lys	β 6	(359, 360, 362, 364)
S	val	β 6	(361, 362, 363)
E	lys	β 26	(159, 367, 368)
Zürich	arg	β 63	(369)
D	glu(NH ₂)	β 121	(164)
δ	thr, asp, ala, ser	β 9, 12, 22, 50	(159, 366)

(31)] and chemical methods [Edmundson & Hirs (32)]. The results of this work together with additional information about the haemoglobins are compiled in Tables II and III.

Human haemoglobin A comprises two α chains (141 residues) and two β chains (146 residues). The data on the amino acid sequence, according to reports from different laboratories (cf. Braunitzer; Hill & Konigsberg), differ only at positions 26-27, 50, and 52 of the α chain and positions 37, 39, 54, 55 of the β chain. These differences represent in most cases only mutual interchanges along the sequence. Of the 151 residues of sperm whale myoglobin only 5 residues at positions 11, 13, 17, 102, and 107 remain undetermined. X-ray diffraction analysis with a resolving potency of 2 Å has proved successful in determining one-third of all residues unambiguously and another third with a high probability [Kendrew (157)]. Seventy per cent of all residues are contained in eight α -helical units (A-H) linked together by irregular sections (157).

Some general conclusions may be drawn from the comparison of the data so far available [Watson & Kendrew (158); Braunitzer *et al.* (153)]: the α and β chains of haemoglobin share identical amino acids at 66 positions, and the two chains have 22 residues in common with myoglobin. At those sites where a peculiar type of amino acid is conditioned by the entire structure an identical residue occurs in both chains of haemoglobin and in myoglobin. Thus the smallest residue in space, glycine, is situated at the places where the B- and E-sections of the α -helix (No. 25 and 59, α chain) closely pass each other. The hydrogen bond, linking tyrosine (No. 40, α chain) with a carbonyl group (No. 94, α chain), stabilizes the G- and H-sections of the α -helix. The phenylalanine residues at positions No. 44 and 46 (α chain), which are

situated outside the α -helical region, apparently stabilize, because of their mutual parallel arrangement, the pyrrole rings of the haem. Histidine No. 58 (α chain) is bound to the iron, and histidine No. 87 (α chain) to the haem.

The cysteine residues No. 104 (α chain) and No. 112 (β chain) are shifted by three residues, most probably by one turn of the α -helix. The positions of proline residues along the chains, with respect to the α -helix sections, suggest that proline is not essential for the chain to turn a corner. The two proteins differ completely in the N-terminal residues of haemoglobin (No. 1-2 in the α chain, No. 1-3 in the β chain) and in the C-terminal peptide of myoglobin (No. 147-151) which do not fall within the α -helical sections. The finding that the α chain of haemoglobin is shorter than the β chain (No. 50-51, α chain; and No. 50-56, β chain) is in accordance with the x-ray diffraction analysis which postulates the occurrence of a small loop at this part of the α chain.

Table III contains data on human haemoglobins of different genetic origin. A large portion of the γ -chain of foetal haemoglobin F ($\alpha^2\gamma^2$) has been resolved by Schroeder (156), Table II; different features of the δ chain of haemoglobin A₂ were demonstrated by Ingram & Stretton (159). Most of the interchanges between pathological haemoglobins (Table III) occur at those sites of the molecule which are without influence on the conformation of the chain. An exception is found in the pathological haemoglobin M whose Fe^{+++} can be reduced to the Fe^{++} form only with difficulty. The interchanges existing in its α chain (No. 58) or β chain (No. 63 or 67) are intimately related to the binding of the iron atom (158). Of the human haemoglobins not recorded in Table III, those examined were haemoglobin G (Honolulu) (160), Ib (161) and D_a (162), differing from haemoglobin A in the α chain; and haemoglobin A'₂₃ (163), D _{β} and D _{γ} = D _{β} (Punjab) (162, 164) differing in the β chain. In addition to foetal haemoglobin F, two additional haemoglobins, "Gower" 1 and 2, were found to be characteristic for the early stages of embryonal development (165). By the use of improved separation techniques, eight components have already been established in human haemoglobin (166, 167, 371). The genetic relationship between human haemoglobins has been reviewed in the papers of Ingram & Baglioni (168, 169, 170) and by Zuelzer & Robinson (370).

The detailed examination of haemoglobins from different species has been limited to horse haemoglobin (171 to 174). This can be separated on carboxymethyl cellulose into two haemoglobins (171) differing only in one chain (173). Braunitzer *et al.* have isolated the two chains from that fraction of haemoglobin which is bound more strongly to carboxymethyl cellulose. The α chain differs from the human α chain in at least 15 residues along the entire length of the chain (171). Oppel has characterized the N-terminal sequence of seven residues in the β chain, which differs from the β chain of human haemoglobin at residues 2 (glu) and 7 (ser) (172). The same tetrapeptide sequence, residues 62 to 65, occurs in the β chain of human, horse, and rat haemoglobin and, moreover, the sequence ala.his.gly.asp(NH₂)

lys exists in horse haemoglobin [Mäsiar (174)]. No differences between foetal and adult horse haemoglobin were revealed when using x-ray diffraction analysis, chromatography, and the fingerprint analysis of peptides [Stockell *et al.* (175)].

Bovine haemoglobin has been separated into the α chain and β chain by electrophoresis [Take (176)] and chromatography [Sasakawa (177)]. Monkey haemoglobins (*Macacus rhesus* and *cynomolgus*) display mobilities identical with that of human haemoglobin A; a component identical in mobility with human haemoglobin AJ and AI has been revealed in some *Cynomolgus* haemoglobin [Tuttle *et al.* (178)]. The heterogeneity of seal haemoglobin (5 components) (180) has been established.

The separation of horse myoglobin on carboxymethyl cellulose yields three components differing in their amino acid sequence [Åkeson & Theorell (179, 181)]. The action of trypsin on acetylated horse myoglobin resulted in the cleavage of bonds involving the carboxyl of arginine and yielded three fragments; two bonds, try.glu, were attacked specifically by N-bromosuccinimide [Dautrevaux *et al.* (182)]. The reported partial structure H.gly.leu . . . try.glu . . . arg.leu . . . arg.ileu . . . try.glu . . . leu.asp (NH₂).phe.gly.OH suggests considerable differences in the amino acid sequence from that of sperm whale myoglobin.

A comparison of seven myoglobins from different species (sperm whale, sei whale, tortoise, horse, seal, porpoise, gentoo penguin) has been carried out by Stockell (183) using the fingerprint technique. He pointed out that sperm whale myoglobin can be separated into two components that differ in a single peptide. All the myoglobins that were compared differed from each other in five peptides at least. Although no differences were revealed between the tertiary structures of sperm whale and seal myoglobin these two proteins differ in at least one half of all the fragments. It is obvious that an identity of amino acid sequence cannot be assumed simply on the basis of far-reaching similarities in tertiary structure.

Ellfolk (184, 185, 186) has separated and analyzed the two basic components of crystalline leghaemoglobin with N-terminal glycine and valine respectively. It is interesting to note that neither cysteine nor methionine is contained.

PEPTIDE HORMONES

The isolation of ACTH and MSH from different species and the synthesis of model peptides have permitted identification of those portions of the molecule which appear to be essential for the hormonal function. Bovine and sheep ACTH resemble each other in structure more than they resemble pig ACTH [Li *et al.* (187, 188)]:

Residue No.	25		33
	↓		↓
bovine	...asp.gly.glu.ala.	glu.asp	ser.ala.glu(NH ₂)...
sheep	...ala.gly.glu.asp.	asp.glu	ala.ser.glu(NH ₂)...
pig	...asp.gly.ala.glu(NH ₂).	asp.glu(NH ₂).	leu.ala.glu.....

After elucidation of the structures of α and β MSH from monkey (189) the structures of MSH from five different species can be compared. Whereas α MSH is identical in all cases distinct differences exist between the structures of β MSH from different species:

		1	2	3	4	5	6	10	20	22
human	(190)	ala.	glu.	lys.	lys.	asp.	glu.arg.pro.asp
monkey	(189)					asp.	glu.arg.pro.asp
pig						asp.	glu.lys.pro.asp
bovine	(191, 192)					asp.	ser.lys.pro.asp
horse						asp.	glu.lys.arg.asp

Human β MSH differs distinctly from all the remaining hormones in its N-terminal peptide (residues 1 to 4).

The synthesis of polypeptides with full ACTH activity represents one of the greatest achievements in the field of organic synthesis of natural sub-

TABLE IV
SYNTHETIC PEPTIDES WITH HORMONE ACTIVITY

No. of Residue in ACTH	Activity	Reference
1 to 24	Full ACTH and MSH activity	(193)
1 to 23	Full ACTH and MSH activity	(194)
1 to 19	30 per cent of ACTH and MSH activity	(195)
4 to 10	MSH and CRF activity	(196)
4 to 10	ACTH inactive; weak MSH activity	
(amide at position 5)	Full CRF activity	(197)

stances. The synthesis of lower units, at the same time, provides information about the structures essential for individual activities (Table IV).

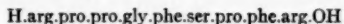
The presence of the acetyl group is more essential for the full activity of α MSH than the two first amino acid residues of the N-terminal extremity [Guttmann & Boissonnas (198)].

Acher (199) has continued his comparison studies on pituitary neuro-hormones of mammals, birds, frogs, and fish. According to his results, vasotocin from fish, frogs, and birds differs from oxytocin in the arginine residue at position 8. On the other hand, vasopressin was found to occur in mammals.

Immunological differences between the growth hormones of human, bovine, and sheep origin have been reported by Moudgal & Li (200). It was observed that of all the preparations so far compared only the growth hormone of the deer was immunologically identical with the bovine hormone. The isolation by countercurrent distribution of polypeptide hormones with calcium-mobilizing and phosphaturic activity from bovine parathyroid glands has been reported by Rasmussen & Craig (201, 202). The molecular weight of 7000 and the amino acid composition of one of these hormones (parathormone B) have been determined. Prolactin has been separated by

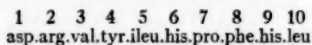
chromatography on Amberlite IRC-50 into three components all of which were found to be equally active [Cole (203)]. The quantitative amino acid composition of pure luteinizing hormone has been determined [Ward *et al.* (204)].

Low-molecular peptides of high biological activity were found to arise from the enzymatic hydrolysis of some components of human plasma. Bradykinin is formed by the trypsin digestion of those plasma proteins which precipitate between 33 and 45 per cent saturation with ammonium sulphate. The structure of bradykinin has been reported by Elliott *et al.* (205) as follows:



The peptide of this formula has been synthesized and found to be fully active, as opposed to the octapeptide which lacks proline at position 7 and was entirely inactive [Boissonnas *et al.* (206, 207, 208), Lewis (209)]. Recently, attention has been attracted to substance P which also exerts smooth-muscle stimulation but differs chemically from bradykinin [Franz *et al.* (210)].

The reaction of renin, from kidney, with horse or pig plasma yields a highly active pressor polypeptide called angiotensin I (211, 212, 213):



An analogous decapeptide bearing valine, instead of isoleucine, at position 5 is formed from bovine plasma. The action of a plasma protease removes the terminal his.leu of angiotensin I, thus giving rise to the octapeptide, angiotensin II. The pressor activity is retained in full; the oxytocic activity, however, increases. The minimum structure required for the two activities to be retained is a hexapeptide sequence (residues 3 to 8). The activity of synthetic angiotensin II equals the full activity of the natural hormone [Arakawa & Bumpus (214)].

The character of erythropoietin, the hormone of erythropoiesis, which is present in plasma of anemic animals is still not clear [Campbell *et al.* (215)]. Although the activity was isolated in the α_1 -glycoprotein fraction with N-terminal serine, this fraction appears not to be the true hormone, but rather a carrier, since a protein of identical properties, but inactive, has been isolated from normal plasma.

A group of substances worthy of special interest, but so far very little explored, is represented by the factors that induce and determine the development of individual organs. Inducing substances of protein character from chicken embryos have been separated and found to exert a specific action on the individual components of the growing embryo [Tiedemann *et al.* (216)]. A growth-control protein factor has been isolated from liver and its activity tested with tissue cultures and by the nematode assay [Sayre *et al.* (217)].

ANTIBODIES

The purification and heterogeneity of γ -globulin have been examined by many authors. New procedures for obtaining the pure γ -globulin fraction by salting out [Volyňskij & Khadjiev (218)] and by precipitation with rivanol [Gubenko (219)] have been described. The purification of specific antibodies by adsorption to their antigens has already been mentioned in the paragraph on the purification of proteins (28). The purification of antibody to β -D-galactosylphenylazo bovine-serum-albumin [Bassett *et al.* (220)] may serve as another example. In the latter case the antibody was precipitated with a heterologous antigen and, after dissociation from the precipitated complex with hapten, the mixture was separated on a Sephadex column.

An interesting problem concerning the existence of a γ -globulin lacking in antibody properties has been investigated by Franěk, Říha & Šterzl (221). They succeeded in isolating an immunologically negative protein, with properties of γ -globulin, by fractionation of the serum of newborn pigs bred under entirely sterile conditions. It is apparent, however, that during the ontogenetic development in response to a vast number of antigens a mixture of antibodies is formed which would display the overall properties of mainly γ -globulin; doubts concerning the heterogeneity of normal γ -globulin could thus hardly be raised. The three different normal γ -globulins revealed by Dray (222) in monkey antibodies to γ -globulin seem to represent the minimum of components really existing.

Fleischer *et al.* (223) have compared the properties of antibodies against azoproteins obtained by coupling bovine γ -globulin with diazotized acyl amines. The analyses of antibodies have shown a practically identical amino acid composition. The authors conclude that the differences between specific antibodies may be attributable to differences in the amino acid sequence or tertiary structure.

The digestibility of antibodies by trypsin and chymotrypsin has been investigated by Gurvich *et al.* (224). A specific rabbit antibody against human and horse serum albumin was hydrolyzed under identical conditions to a lower degree than the unspecific rabbit γ -globulin.

A number of theories considering the structural conditions of antigenicity have been advanced. To one of them the essential part in the process is played by aromatic amino acid residues. Haurowitz (20) related antigenicity to the rigidity of the molecule. This opinion has been given further support by the experiments of Sela & Arnon (225, 226). Gelatin contains neither tyrosine nor tryptophan and is not an antigen. The authors have shown that antigenicity of polytyrosyl-gelatins to be specific and to increase with the tryosine content. This might indicate the importance of the aromatic component. However, analogous polycyclohexylanalyl gelatins prepared by these authors were also found to be powerful antigens. In view of these results they have concluded that the enhancement of the antigenicity is brought about by an increase of the rigidity of the molecule because of the attachment of substituents large in size and not merely to their aromatic character.

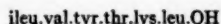
Interest in the structural investigation of antibodies has been strongly stimulated by the work of Porter (227, 228) who has demonstrated that specific antiovalbumin when digested by papain gives two fragments which retain the power to combine with the antigen and a third fragment which has antigenic properties but is not bound by the antigen. The cleavage by papain has been explored by many other authors (229, 230, 231) and the results of Porter were confirmed. Gitlin & Merler have been able to demonstrate from a comparison of partial hydrolysates that two of the three components released by papain are most likely of identical structure.

A bivalent antibody of molecular weight approximately 106,000 giving two univalent antibodies of approximately half the molecular weight, after reduction of one disulphide bond by 2-mercaptoethylamine, has been prepared by Nisonoff *et al.* (232, 233, 234) from antiovalbumin treated with pepsin. Franěk (63) has cleaved the disulphide bonds of γ -globulins from different species by conversion into S-sulpho derivatives. The molecular weight of pig, bovine, horse, and human γ -globulin dropped to one fourth, the molecular weight of rabbit γ -globulin to one half.

The digestion of human γ -globulin with papain and the isolation of glycopeptides with the sequence $\text{glu}(\text{NH}_2).\text{glu}(\text{NH}_2).\text{asp}(\text{NH}_2).\text{tyr}.\text{glu}.\text{asp}$ attached by the carboxyl group of the C-terminal aspartic acid to the polysaccharide have been reported by Rosevear & Smith (235).

MILK AND EGG PROTEINS

α -Lactalbumin.—A single polypeptide chain in α -lactalbumin (mol wt 16,300) is evidenced, among others, by the fact that the molecular weight is not changed by the reduction of disulphide bridges [Weil & Seibles (236)]. The N-terminus is occupied by glutamic acid (glutamine) and the C-terminus by the peptide,



α -Lactalbumin heated at 100° C for a short time or deprived of its C-terminal leucine could be crystallized [Wetlaufer (237)].

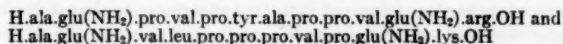
β -Lactoglobulin.— β -Lactoglobulin is a mixture of two proteins, A and B, of identical amino acid composition [Townend *et al.* (64), Dawson (238)]. At pH 5, β -lactoglobulin exists in dimer form AA or BB; dissociation into the monomer A or B of molecular weight 18,000 occurs at pH 2. The formation of dimer hybrids AB does not take place. The milk of some cows contains only the type AA or BB or the mixture of both. The origin of both types seems thus to be controlled only by one gene (64). Studies of the secondary structure of β -lactoglobulin by Tanford & De (239) provide evidence of a high percentage of α -helices in aqueous-organic solutions of the protein, contrary to water solutions.

A comparative examination of buffalo milk and cow milk performed by Sen & Sinha (240) has shown differences in the electrophoretic behaviour of both the α -lactalbumins and the β -lactoglobulins.

Casein.—A systematic fractionation of casein has so far yielded four

different proteins, the α -, β -, γ -, and κ -casein which differ considerably in composition (241, 242, 243). The primary reactions in the precipitation of casein with rennin involve exclusively κ -casein [Wake & Baldwin (241)].

Two basic peptides with streptogenin activity have been isolated from tryptic hydrolysate of casein by Baudet *et al.* (244):

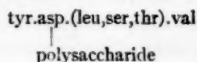


If the tryptic hydrolysate is subsequently digested with subtilisin the growth-promoting activity towards *Lactobacillus casei* is increased. It is always higher than the activity of the total hydrolysate or of the intact protein [Mosolov & Skarlat (148)]. A glycopeptide has been isolated from the peptic digest of casein, which in its amino acid composition is apparently identical with the peptide released from casein by rennin. The peptide is characterized by a high proline content (11 per cent) [Habermann *et al.* (245)]. Pure phosphopeptides have been isolated from tryptic and peptic digests of γ -casein (246). These results, together with the previous data on phosphopeptides from α - and β -casein (247, 248), contribute to our present state of knowledge of the phosphorus bond in phosphoproteins.

Lysozyme.—The chymotryptic cleavage (249) of egg-white lysozyme and the hydrolysis of reduced lysozyme substituted with iodoacetic acid (250) have enabled Jollès to report further details of the primary structure. The sequence of 46 residues of the N-terminal portion of the chain has been determined unequivocally; the five fragments described by the authors account for 128 residues. The C-terminal peptide reported by these authors is not in agreement with the data of Ando *et al.* (251). Lysozyme of molecular weight 21,000 has been isolated from T2 phage by Katz & Weidel (252).

Ovalbumin.—Based on his titration experiments, Belicer (253) has assumed ovalbumin to represent a mixture of two different forms with one and two disulphide bonds respectively. The photo-oxidation of ovalbumin at pH 8.1 to 8.3 brings about a rapid oxidation of histidine, cystine, a part of tryptophan, and tyrosine; at pH 5.4 to 5.6 histidine is not oxidized [Kotkova *et al.* (254)]. Oxidation by periodic acid results in the complete destruction of cystine and tryptophan [Maekawa & Kitazawa (352)].

Structural investigation of ovalbumin, predominantly of the mode of linkage of the sugar components, has been continued by Neuberger *et al.* (255, 256) and Kaverzneva (257, 258). It may be considered proved that the N-terminus is acetylated and that the sugars are bound via aspartic acid in the following sequence of residues:



Ovomucoid.—Jevons (259) succeeded in further purification of ovomucoid on TEAE-cellulose. Papain removes from ovomucoid a fragment which is homogeneous after isolation on DEAE-cellulose and has a molecular weight of 6500 [Morton & Deutsch (18)].

Egg yolk proteins.—Proteins of egg yolk have been fractionated systematically to α -, β -, γ -livetins, α -, β -lipovitellins and phosvitin [Cook *et al.* (260, 261)]. The terminal groups of vitellin and vitellinins have been determined by Neelin & Cook (262). Undoubtedly the fractionation of the proteins of egg yolk cannot be considered as being completed since, e.g., phosvitin can further be separated into two components that differ in amino acid composition and metal content [Connelly & Taborsky (263)]. Phosvitin of egg yolk is identical with phosvitin from hen serum. It has a molecular weight of 40,000, and contains 31 per cent of serine and traces of tryptophan; the N-terminal amino acid is alanine [Mok *et al.* (264)].

FIBROUS PROTEINS

Fibrinogen.—Two peptides are released during the fibrinogen-to-fibrin conversion. The identity of the optical rotation of fibrinogen and the fibrin monomer led Kay & Marsh (265) to the conclusion that the fibrinopeptides are not arranged in an α -helical structure. The structure of fibrinopeptides from bovine fibrinogen has been determined by Sjöquist *et al.* (266). The fibrinopeptide A contains 18 amino acid residues, the fibrinopeptide B, 20 residues including tyrosine-O-sulphonic acid.

Collagen.—The structural investigation of procollagen has been continued by Grassmann *et al.* (267, 268). From a tryptic digest they obtained mostly peptides consisting of three parallel chains always N-terminated with glycine and C-terminated with arginine or lysine. Collagenase has been found to attack, predominantly, bonds involving the amino group of glycine. Ninety-four per cent of all proline in collagen is bound in the sequences gly.pro.gly, gly.pro.ala, and gly.pro.(OH)pro. Previous studies in this field have been reviewed by Grassmann (269).

Fibroin.—The quantities of amino acids in fibroins from 76 different species have been compared by Lucas *et al.* (270). Several lower peptides resulting from chymotryptic cleavage of fibroin have been characterized [Ziegler & LaFrance (271)].

Myosin.—Tropomyosin from the muscle of *Pinna nobilis* yielded a homogeneous tryptic core of approximately half the molecular weight (about 8500), which did not differ from the parent protein in amino acid composition [De Milstein & Bailey (272)]. The N-terminal group of this core consists of glutamic acid, as in rabbit tropomyosin [Saad & Kominz (273)].

Miscellaneous.—An interesting amino acid pattern is encountered in the fibrous proteins of spider silk in which cysteine is replaced by cysteic acid [Fischer & Brander (274)]. A new rubber-like protein differing from collagen, fibroin, and elastin has been found in insects and termed resilin [Bailey & Weis-Fogh (275)]; proteins isolated from the shells of mollusca were called conchagens [Stegemann (276)].

STRUCTURE OF OTHER PROTEINS

Insulin.—The relationship between structure and function in insulin has been further examined. A terminal heptapeptide has been removed from

insulin by trypsin in the experiments of Young & Carpenter (277). In contrast to a previous observation (278) the residual protein lost all activity. Dixon & Wardlaw (279) have separated the two chains of insulin as S-sulphonates. One to two per cent of activity was regenerated when the derivatives were mixed and thiol added.

Ribonuclease.—After elucidation of the complete structure of ribonuclease attention has predominantly been focused on the relationship between structure and function, especially by Richards & Vithayathil (280, 281, 282), Moore *et al.* (283, 284), and Anfinsen *et al.* (285, 286). Obviously, the ribonuclease activity is conditioned by the specific structure of 20 residues in the N-terminal portion of the molecule (280, 281), by the existence of aspartic acid at position 121 (287), histidine at position 119 (283), and lysine at

TABLE V
ACTIVE SITES OF ENZYMES REACTIVE TO ORGANOPHOSPHATES

Enzyme	Sequence	Reference
Chymotrypsin	gly.asp.ser.gly.gly.pro.leu	(345)
Trypsin	gly.asp.ser.gly.pro.val.cys	(346)
Elastase	gly.asp.ser.gly	(72)
Thrombin	gly.asp.ser.gly	(347)
Liver aliesterase	gly.glu.ser	(348, 349)
Pseudocholinesterase		
Subtilisin	thr.ser.met.ala	(350)
Phosphoglucomutase	thr.ala.ser.his.asp	(296)
Phosphorylase A	lys.glu(NH ₂).ileu.ser.val.arg	(351)

position 41 (288). Native, completely reduced, ribonuclease and reoxidized ribonuclease possess identical secondary and tertiary structures. Hence, all information underlying higher structures is contained in the primary structure [White *et al.* (289)]. The comparison of structures of ribonucleases from different species holds promise of providing very interesting data. So far, new ribonucleases have been isolated from takadiastase (290), *Euglena gracilis* (291), *Bacillus subtilis* (292), spinach (293), and guinea pig pancreas (294).

Esterases.—The structure of the active sites of nine esterases has been solved by studies of their reaction with organic phosphorofluoridates. A comparison is presented in Table V.

From the work of Milstein (295), the structure of the active site of rat liver phosphoglucomutase is identical with the sequence around the active site of phosphoglucomutase from rabbit muscle [Milstein & Sanger (296)].

Serum albumin.—Several authors have investigated the heterogeneity of human serum albumin (297, 298, 299) and concluded that the protein mainly represents a mixture of a monomer and a dimer accounted for by the free

SH-group of the monomer. Richard *et al.* (300) have isolated from a chymotryptic hydrolysate of bovine serum albumin peptides ranging in molecular weight from 3200 to 6800; all these peptides formed precipitates with anti-serum against the parent serum albumin. Grannis (38) has obtained a dodecapeptide by specific hydrolysis of human serum albumin with dilute acid, and determined its structure.

Plant viruses.—The complete structure of the protein component of the tobacco mosaic virus has been determined in Berkeley and in Tübingen [Schramm *et al.* (301); Fraenkel-Conrat *et al.* (302)]. A comparison of the two proposed formulae reveals complete agreement of the sequence of 157 amino acid residues except for three positions with interchanged sequences (-pro.thr-, -pro.ser-, leu No. 29) and the localization of amides.

A comparison of protein components of two different strains of tobacco mosaic virus has been reported by Wittmann & Braunitzer (303, 304). From the characterization of peptides formed by tryptic digestion, the authors conclude that of the 157 residues 44 are different in the two strains. Comparative studies considered from the genetic aspect are presented in the report of Friedrich-Frekša (305).

Apart from tobacco mosaic virus, structural investigations of the protein component have hitherto been largely centered on the turnip yellow mosaic virus consisting of monomers of molecular weight 21,300 [Harris & Hindley (61)]. As in the tobacco virus the N-terminal extremity of the molecule is acetylated; the sequences of thirteen residues of the N-terminal portion and five residues of the C-terminal portion have been elucidated.

Plant proteins.—The terminal groups of several proteins from plant seeds have been determined. The N-terminus is occupied by glutamic acid in glutelin from barley [Meltjeva (306)], by glycine and leucine in legumin, and by aspartic acid and serine in vicilin (both proteins originating from peas) [Weintraub & Hofmann (307)]. The C-terminal group of gliadin is glutamine; edestin is C-terminated with asparagine [Veksler *et al.* (308)]. A number of peptides have been isolated from partial hydrolysates of edestin [Karadžova *et al.* (309)].

Histones.—Histones have been fractionated on paper (310), in agar jelly (311), and on carboxymethyl cellulose (312). The arginine-rich fraction of histone was digested with the *Streptomyces griseus* protease (313) and trypsin (314). Although the average content of basic amino acids in histone accounts for one third of all residues, they do not occur with regular frequencies [Phillips & Simson (314)].

Clupeine.—Ando *et al.* (315) were successful in isolating a homogenous clupeine Z from a mixture of clupeines and determined its complete structure. The structure of this first clupeine so far solved may be presented schematically (arg = R, neutral amino acids = N) as follows: $\text{NR}_4\text{NR}_2\text{N}_2\text{RN}_3\text{NR}_2\text{N}_2\text{R}_4\text{NR}_4$.

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AMINO ACID METABOLISM^{1,2}

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For the major amino acids, although certain islands of obscurity remain, much of the descriptive detail of pathway metabolism is known. Interest in amino acid metabolism has therefore turned increasingly from the reaction sequences themselves to the mechanism of individual reactions, the analysis of physiologic and genetic controls, and the biochemical details of a growing list of human metabolic derangements. This review inescapably deals with some of these developments as they pertain to individual amino acids; its most responsible emphasis, however, is on metabolic reactions concerned with the synthesis, interconversion, and degradation of amino acids as such.

METABOLIC ASPECTS OF NEW AMINO ACIDS

The recent appearance of an encyclopedic treatise on amino acid chemistry by Greenstein & Winitz (1) is a notable event. These volumes list over 90 naturally occurring nonprotein amino acids. Additions to the list during the past year, either as newly discovered substances or newly verified structures, are summarized in Table I. Metabolic information has quickly followed the discovery of certain of these new compounds. β -(Pyrazolyl-N)-L-alanine is enzymatically degraded by *Pseudomonas* extracts to yield pyrazole, pyruvate, and ammonia (23). Isovalthine levels in cat urine are increased by feeding leucine (24). Agaritine is cleaved at the hydrazide bond by an enzyme derived from those mushrooms that contain the compound (13). The γ -hydroxyarginine of sea anemones is decarboxylated by *Escherichia coli* preparations (25) and hydrolyzed by beef liver arginase (26). Aubert *et al.* (27) have published a full report of the isolation and characterization of N-succinyl-L-glutamate from *Bacillus megaterium* and other spore-formers; the compound accumulates when growth is interrupted and may therefore have physiological significance related to sporulation.

TRANSAMINATION

Mechanism.—The binary mechanism of enzymatic transamination (28), consistent with the recent findings of Jenkins & Sizer (29) with substrate

¹ The survey of literature pertaining to this review was completed in September 1961.

² The following abbreviations will be used: AMP (adenosine monophosphate); DOPA (3,4-dihydroxyphenylalanine); DOPamine (3,4-dihydroxyphenylethylamine); FAD (flavin adenine dinucleotide); NAD (nicotinamide adenine dinucleotide); NADP (nicotinamide adenine dinucleotide phosphate); NADH₂ (nicotinamide adenine dinucleotide, reduced form); NADPH₂ (nicotinamide adenine dinucleotide phosphate, reduced form); PP (inorganic pyrophosphate); PRPP (5'-phosphoribosyl-pyrophosphate).

quantities of highly purified glutamic-aspartic transaminase, is supported by similar studies of Lis *et al.* (30). An enzyme of like specificity, purified from cauliflower florets by Ellis & Davies (31), also behaved consistently with the binary mechanism (32). One discrepancy—the apparent unreactivity of the glutamine and asparagine transaminases with a number of common

TABLE I
NATURALLY OCCURRING AMINO ACIDS



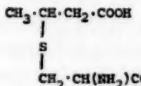
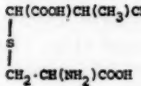
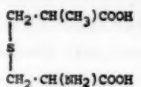

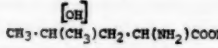



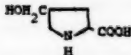
Structure	Name, Source, Comment	
 COOH $\text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	<i>m</i> -Carboxyphenyl-L-alanine, from iris bulb.	(2)
 $\text{N-N} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	β -(Pyrazolyl-N)-L-alanine, from watermelon seeds. See text.	(3)
 $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{COOH}$ S $\text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	S-(β -carboxyisopropyl)-L-cysteine, from <i>Acacia</i> seeds.	(4)
 $\text{CH}(\text{COOH})\text{CH}(\text{CH}_3)\text{CH}_3$ S $\text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	S-(isopropylcarboxymethyl) cysteine ("Isovalthine") from human urine. See text.	(5)
 $\text{CH}_2 \cdot \text{CH}(\text{CH}_3) \text{COOH}$ S $\text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	S-(-2-methyl-2-carboxyethyl) cysteine, from human urine.	(6)
$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	Ethionine, from bacteria.	(7)
 COOH H_2N	4-Aminopipicolinic acid, from <i>Strophanthus</i> .	(8)
 $\text{CH}_3 \cdot \text{CH}(\text{CH}_3) \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	Hydroxyleucine (position of OH not established), from <i>Deutsia</i> .	(9)
$\text{CH}_3 \cdot \text{CH}_2 \cdot \text{NH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	N-Ethyl-L-asparagine, from squirting cucumber.	(10)

TABLE I—(Continued)

Structure	Name, Source, Comment
 $\text{CH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{COOH}) \text{CH}_2 \cdot \text{COOH}$	N-(Indole-3-acetyl) aspartic acid, from tomato. (11)
$\text{H}_2\text{N} \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	Homocitrulline, from human urine. (12)
 $\text{NH} \cdot \text{NH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	β -N-(γ -L-Glutamyl)-4-hydroxymethylphenylhydrazine ("agaritine") from mushrooms. See text. (13, 14)
 $\text{NH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	N-(γ -L-Glutamyl)-4-hydroxyaniline, from mushrooms. (15)
$\begin{array}{c} \text{CH}(\text{COOH})\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2\text{NH}_2 \\ \\ \text{NH} \\ \\ \text{CH}(\text{COOH})\text{CH}_3 \end{array}$	D-Lysopine, from crown galls. (16, 17)
$\begin{array}{c} \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH} \\ \\ \text{NH} \\ \\ \text{CH}(\text{COOH})\text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} \end{array}$	Saccharopine, from yeast. (18)
$\text{H}_2\text{N} \cdot \text{C}(:\text{NH})\text{NH} \cdot \text{CH}_2 \cdot \text{CH}(\text{OH})\text{CH}_2 \cdot \text{CH}(\text{NH}_2) \text{COOH}$	γ -Hydroxyarginine, from sea anemone. See text. (19)
	4-Hydroxymethyl-L-proline, from apples. (20, 21)
$\text{CH}_3 \cdot \text{CH}(\text{OH})\text{CH}(\text{NH}_2) \text{COOH}$	D-Allothreonine, from <i>Mycobacteria</i> . (22)

amino acids, even though their corresponding α -keto acids were known to be active as substrates—has been resolved by Braunshtein's report (33) that the purified amide transaminases (34) do catalyze the slow amination of several α -keto acids by a number of monocarboxylic amino acids. Direct demonstration has been made by Hiller & Walker (35) of the expected transaminase-catalyzed exchange reaction between aspartate and oxalace-

tate; the rate of exchange is faster than the rate of transamination between the glutamate and aspartate skeletons.

β -Dissociation of the Schiff-base intermediate, as an obligatory step in transaminase action, was suggested by Oshima & Tamiya (36) on the basis of transaminase-catalyzed exchange of the β -hydrogens of alanine in D_2O . This interpretation was criticized by Harley-Mason (37) on the grounds that spontaneous exchange of the β -hydrogen would be expected for a Schiff-base complex between alanine and pyridoxal, so that no revision of presently accepted views is required.

Banks (38) points out that, although the spontaneous conversion of keto-oxalacetate to its enol form is a relatively slow process, the enol form (identified as that with high absorption at 280 $m\mu$) appears without lag as an enzymatic product of aspartate and α -ketoglutarate, even with high levels of purified transaminase. Since there is considerable evidence for a Schiff-base intermediate, requiring keto-oxalacetate as the enzyme-bound form, accelerated conversion to the enol needs explanation. *Erythro- β -hydroxy aspartic acid was found by Jenkins (39) to react with the pyridoxal form of purified glutamate-aspartate transaminase, forming a deep yellow complex believed to be an enzyme-substrate intermediate.*

Distribution and specificity.—The extensive clinical interest in serum transaminase determinations makes the release and subcellular distribution of these enzymes matters of practical concern. Chilled animals show an elevation of serum glutamate-aspartate transaminase without histological evidence of visceral necrosis (40), thus supporting the suggestion that enzyme leakage may occur through functionally impaired membranes. Eichel & Bukovsky (41) have confirmed earlier reports that the largest fraction of glutamate-aspartate transaminase is found in liver mitochondria; the enzyme is activated by procedures, such as supersonic vibration, which break mitochondria (42). The number and specificity of the transaminases of rat liver were studied by Hammar (43) with the conclusion that at least three distinct enzymes must be postulated.

The glutamate-aspartate transaminase of cockroach (*Periplaneta*) fat body was purified by McAllan & Chefurka (44) with results closely similar to those for the corresponding enzyme of pig heart. Segal & Beattie (45) were unable to detect differences in purification behavior or in kinetic properties between the preparations of liver glutamic-alanine transaminase, purified 200-fold from untreated or from corticoid-treated rats; in the latter animals, as had been observed earlier (46), the specific activity of the enzyme in crude homogenates was fivefold greater than that from untreated animals.

OXIDATION

Mammalian D-amino acid oxidase, crystallized by Kubo *et al.* (47), was reported by them to contain iron, presumed to participate in the catalytic mechanism. Further purification of the enzyme by Massey *et al.* (48) yielded more active, iron-free crystals. Kinetic and spectrophotometric studies (48)

of this preparation suggested that in the normal catalytic mechanism enzyme-bound FAD is oxidized and reduced only to the semiquinone. Further studies of similar preparations by Charlwood *et al.* (49) indicated that the basic unit of D-amino acid oxidase is a protein of 45,700 molecular weight which can polymerize to at least the tetramer. Excess FAD appeared to encourage polymerization.

Yagi and associates (50) have obtained a crystalline preparation of D-amino acid oxidase containing equimolar proportions of benzoate and FAD. Since a step in the purification of the enzyme by both of the earlier groups (47, 48) involved heating in the presence of stabilizing concentrations of benzoate, Yagi *et al.* (50) suggest that these earlier crystalline preparations may also represent enzyme-benzoate complexes.

The recent purification of L-amino acid oxidase from snake venom by Wellner & Meister (51) has permitted more detailed studies of this enzyme as well. As with D-amino acid oxidase (48), kinetic observations (52) also support a mechanism in which the two moles of FAD per mole of protein (51) are each half-reduced by a mole of substrate. The well-known inhibitory effect of excess substrate has been interpreted both by Wellner & Meister (52) and by Marcus & Feeley (53) as resulting from complete reduction of FAD by excess substrate; doubly-reduced FAD is postulated to be more slowly reoxidized than half-reduced FAD. This conclusion was supported by the reduced inhibitory action of excess substrate in the presence of high O_2 concentration (53); more quantitatively, agreement was found between experimental observations and a rate equation derived from this concept of substrate inhibition (52).

DECARBOXYLATION AND RACEMIZATION

The steric fate of the entering proton during enzymatic decarboxylation of an amino acid was investigated by Belleau & Burba (54) using the two deuterium enantiomers, R- and S- α -D-tyramine. Because these are oxidized at appreciably different rates by monoamine oxidase, it was possible to identify the configuration of the tyramine formed enzymatically from tyrosine in D_2O and thus to show that the reaction proceeds with retention of configuration, the entering proton (or deutron) positionally replacing the carboxyl group.

A new group of amino acid decarboxylases, derived by Seaman (55) from a pseudomonad, differ from the familiar decarboxylases of *E. coli* (56) in their appearance during growth in alkaline rather than acid media. Whole cells caused the release of CO_2 from 18 amino acids including the imino acids, proline and hydroxyproline—a point of interest in view of Braunshtein's generalization (57) that pyridoxal phosphate enzymes require an unsubstituted amino group in their substrates in order to accommodate a Schiff-base intermediate.

Glutamic acid racemase of *Lactobacillus fermenti*, purified about 500-fold, is claimed in a preliminary report by Tanaka *et al.* (58) to show a flavin

spectrum and to lose activity with riboflavin, restorable, however, by FAD; the inactivation and reactivation data presented do not seem conclusive. Unlike the enzyme recently purified by Glaser (59) from *L. arabinosus*, carbonyl reagents such as hydroxylamine failed to inhibit. The possibility remains that a prosthetic group other than, or in addition to, pyridoxal phosphate is a cofactor of certain amino acid racemases.

GLUTAMIC ACID AND γ -AMINOBUTYRIC ACID

Glutamic acid.—Recognition of the wide substrate specificity of glutamic dehydrogenase and the interrelations between inhibitor action, substrate specificity, and state of aggregation have greatly stimulated interest in the structure and mechanism of this enzyme. Fisher's studies indicate that $(\text{NH}_4)^+$ is bound competitively with glutamate (60), and that alanine is unequivocally a substrate for glutamic dehydrogenase and does not arise from pyruvate via "transdeamination" (61). Fisher's conclusion that alanine is bound at the same site as glutamate is not supported by the kinetic observations of Tomkins *et al.* (62). Frieden (63) has observed that NADPH_2 -binding as measured by fluorescence enhancement shows different characteristics from binding as determined by enzyme activity, and therefore suggests that NADPH_2 is also bound at catalytically inactive sites.

In the studies of Yielding & Tomkins, the disaggregation of the enzyme promoted by inhibitory steroids (64) and by NADH_2 (62) was prevented by AMP, ADP, and by certain amino acids (65). The estrogen-inhibited enzyme catalyzed an accelerated reaction between alanine and pyruvate, and since other disaggregating agents stimulated the dehydrogenation of alanine while inhibiting that of glutamate, it is suggested that dissociation of the enzyme promotes alanine dehydrogenation while aggregation promotes glutamate as a substrate. Irradiation of the enzyme was found by Adelstein & Mee (66) to decrease the V_{max} and increase the K_m for glutamate and α -ketoglutarate and to result in dissociation into more slowly sedimenting components. Possible enhancement of the alanine dehydrogenase activity in fragments obtained by this technique was not reported.

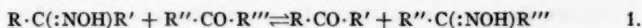
Further examples of genetically altered forms of glutamic dehydrogenase from *Neurospora* were described by Fincham & Bond (67). The existence of two distinct glutamic dehydrogenases, one NAD-linked and one NADP-linked, has been noted by Sanwal both in *Fusarium* (68) and *Neurospora* (69), and extends the earlier finding of Holzer *et al.* (70) of distinct forms of the enzyme in yeast. The role of duplicate enzymes may be related to selective biosynthetic and degradative functions of the two forms, since the NADP-enzyme of *Fusarium* is relatively abundant early in the growth cycle, while the NAD-enzyme increases only later (68).

Wilson & Koeppel (71) found that both D- and L-glutamate are utilized by the intact rat through multiple pathways. D-Pyrrolidone carboxylate is a product of D-glutamate both in the intact rat and in tissue slices, while labeled L- or D-glutamate, administered intracecally, yields labeled acetate

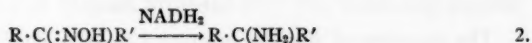
and pyruvate. The mesaconate-citramalate pathway, presumably effected by the intestinal flora, could catalyze both racemization and the fermentation reactions described by Barker (72) and may be invoked by way of explanation.

Halpern & Grossowicz (73) confirmed the earlier finding that glutamyl transferase in *Mycobacterium phlei* is not associated with glutamine synthetase activity. A further paper by Meister *et al.* (74) on the homogeneous glutamine synthetase from sheep brain describes a sedimentable complex formed from enzyme, ATP, and glutamate (in the absence of NH_3), which can be decomposed by heat to yield pyrrolidone carboxylate, or which yields glutamine on treatment with NH_3 .

The transoximase reaction (Reaction 1), described earlier by Yamafuji



(75), was also reported from chicken liver (76), in which it was presumed to couple with an "oximase" step (Reaction 2) since α -ketoglutarate incubated



with acetoxime, NADH_2 , and FAD yielded glutamate. Data presented, however, do not eliminate alternative possibilities such as the reductive release of NH_3 from acetoxime and the subsequent action of glutamic dehydrogenase.

γ -Aminobutyric acid.—In a detailed study of the developing optic lobe of chick brain, Siskin *et al.* (77) found parallel increases in γ -aminobutyrate, glutamic decarboxylase, and γ -aminobutyrate transaminase. Although the blood-brain barrier is impermeable to systemically injected γ -aminobutyrate in adult animals, it was possible to raise the brain level of γ -aminobutyrate in young chicks in this manner. Observations of McKhann & Tower (78) suggest that in mitochondrial preparations from cerebral cortex, competition for NAD may determine which of the two pathways of α -ketoglutarate oxidation is favored, the direct oxidative pathway to succinate, or that involving transamination with γ -aminobutyrate to succinic semialdehyde and oxidation of the latter.

Wallach (79) has described the action of aminooxyacetic acid as an inhibitor of γ -aminobutyrate transaminase, both of *E. coli* and mammalian brain. This compound has a K_i of about $8 \times 10^{-6} M$ (for the *E. coli* enzyme) and is active in mammals *in vivo* at relatively low dosage (20 to 100 mg per kg), leading both to elevated γ -aminobutyrate levels in brain and to a general sedative and muscle-relaxing effect.

A preliminary communication by Sano & Roberts (80) describes the tissue-specific binding of γ -aminobutyrate by a sedimentable fraction from brain homogenate. Further purification of the binding substance encountered the difficulty that various potential solubilizing treatments all destroyed the binding capacity.

An additional derivative of γ -aminobutyrate isolated from brain is homocarnosine, γ -aminobutyryl-L-histidine, characterized by Pisano *et al.* (81) and present in beef brain at an estimated level of 4 mg per 100 g. The compound could not be found in tissues other than brain. However, appreciable homocarnosine was found in rat muscle after feeding γ -aminobutyrate in order to achieve measurable levels of the latter compound in muscle (82). Studies by Frontali (83) of the nervous tissue of the bee indicate free γ -aminobutyrate levels comparable with those of mammalian brain, together with glutamic decarboxylase. The inhibiting transmitter substance of crustacean nerve, however, was found by Florey & Chapman (84) to be chromatographically distinguishable from γ -aminobutyrate, although the two substances have similar pharmacologic action on the stretch receptors.

A new fermentative pathway for γ -aminobutyrate in a *Clostridium* involves conversion to acetate and butyrate via the successive intermediates succinic semialdehyde, γ -hydroxybutyrate, and vinylacetate (85).

ASPARTIC ACID

The aspartase of *Bacillus cadaveris* has been purified about fifteenfold by Wilkinson & Williams (86) who thus separated the enzyme from fumarylase and permitted determination of K_m for the deamination (approximately 10^{-2} at 29° in the direction of deamination). A preliminary description of crystalline β -methylaspartase, which catalyzes deamination to mesaconate, has been reported by Bright & Ingraham (87).

Woolley (88) has shown that β -methylaspartate is a growth inhibitor of *E. coli*, apparently acting by competition with aspartate utilization for pyrimidine synthesis. Although the carboxylation of β -methylaspartate and subsequent cyclization might conceivably represent an alternate pathway of thymine synthesis, β -methylaspartate had no growth activity for a thymine-deficient strain of *E. coli*.

A striking application of the principle of derepression to enzyme purification was made by Sheperdson & Pardee (89) in the purification of aspartate transcarbamylase from *E. coli*. By growing an earlier-blocked mutant on a sequence of limiting uracil and dihydro-orotate, the enzyme in crude extracts was obtained with a specific activity 1000 times that of comparable wild-type extracts; this permitted crystallization of an essentially pure protein after only a fourteen-fold further purification. The enzyme was calculated to represent 7 per cent of cell protein in such derepressed cultures.

Increasing knowledge of control mechanisms in microbes was also applied by Stadtman *et al.* (90) to the analysis of multiple pathways leading from aspartate to lysine, threonine, or methionine, respectively. In *E. coli*, the aspartokinase reaction, a common first step toward all three products, is represented by at least two separable enzymes, one of which is inhibited by lysine and the other by threonine. The lysine-inhibited enzyme is also repressed by lysine but not by threonine. In yeast, in which lysine does not

arise from aspartate, only a threonine-inhibited aspartokinase was demonstrable. The existence of multiple enzymes for such a common first step would permit more discriminating feed-back control of the appropriate enzyme by the corresponding individual product.

The chemical synthesis and ion-exchange separation by Kornguth & Sallach (91) of the two diastereomeric racemates of β -hydroxyaspartate permitted the testing of each racemate enzymatically, with the conclusion that only *erythro*- β -hydroxyaspartate is a substrate for transamination with α -ketoglutarate by sheep-brain, or for N-carboxylation by carbamyl phosphate with a liver preparation.

Asparagine is synthesized from aspartate in rat liver slices with glutamine or NH_3 as additions, but isotopic measurements by Lerman & Mardashev (92) do not favor direct amide transfer, since the specific activity of the asparagine was considerably less than that of the aspartate. α -Ketosuccinamate was converted to asparagine in this system without reduction in specific activity, presumably via transamination with glutamine—a reaction described in earlier work of Meister & Fraser (93). The amidation reaction itself remains obscure.

An asparagine synthetase, purified 100-fold from yeast, catalyzes a reaction analogous to that of glutamine synthetase [AlDawody & Varner (94)].

ALANINE

The alanine dehydrogenase of *B. cereus* spores, of special interest because of its relationship to spore germination, has been purified about sixtyfold by O'Connor & Halvorson (95). Studies with the partly purified enzyme (96) indicate rather wide substrate specificity, requiring a free carboxyl group, a free amino group, and an α -hydrogen.

The variable level of alanine dehydrogenase in *Rhizobium* strains is reported by Browsers (97) as not correlating with the nitrogen-fixing efficiency of the various strains.

A full description by Hayaishi *et al.* (98) of the α -alanine- β -alanine transaminase of *Pseudomonas* has appeared. The enzyme is relatively inactive with α -amino acids other than alanine, and is inactive with α -ketoglutarate. Malonic semialdehyde, the transamination product of β -alanine, is inducibly oxidized to acetyl CoA. The transaminase provides a possibly significant biosynthetic route for β -alanine.

A further study has appeared (99) of mammalian preparations which in earlier Russian literature were reported able to catalyze the direct amination of pyruvate to alanine. Starch electrophoresis of rat kidney preparations separated the alanine-synthesizing activity from transaminases but not from glutamic dehydrogenase. In view of the now-recognized substrate range of the latter enzyme, the possibility seems real that mammalian alanine-synthesizing fractions represent glutamic dehydrogenase or its disaggregated forms (see p. 178).

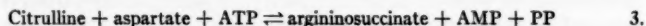
ARGININE AND UREA SYNTHESIS

Arginase.—Bach & Killip (100) have published a detailed report of previous preliminary findings that the arginases crystallized from sheep, horse, and beef liver are very similar in purification behavior and kinetic properties. Close similarity or identity has also been reported by Cabello *et al.* (101) for the enzymes purified from human liver and human erythrocytes. A protein fraction from chicken heart that stimulates partly purified beef-liver arginase has been purified 300-fold by Ceska & Fisher (102); information concerning its mode of stimulation is not available.

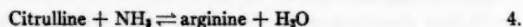
An earlier report by Rogers (103) that the arginase of rabbit epithelium is induced by Shope papilloma virus has been viewed by Rothberg & Van Scott (104) as a consequence of the epidermal hypertrophy of the virus-induced papillomas, since the latter authors find that the arginase of normal rabbit skin is virtually confined to the epidermis. The localization of arginase primarily in the granulocytes and not in the mononuclear or blast cells of peripheral blood is reported by Tanaka & Valentine (105).

Ashida & Harper (106) have added some new observations to the earlier-recognized correlation of liver arginase activity with the protein content of the diet: arginase levels increase in rats within a day after the casein content of the diet is increased, and correlate closely with urinary urea. The arginase activity of a number of cell strains *in vitro* is stimulated by arginine but only if RNA is also added to the medium (107).

Urea cycle.—Studies by Ratner and associates deal with the energetics (108) and mechanism (109) of the following reaction catalyzed by argininosuccinate synthetase:



A pH-independent expression for K_{eq} was calculated (108), from which the ΔF° for the formation of the guanidino group has the value 8.6 kcal:



Purification of the enzyme, to remove adenylate kinase and inorganic pyrophosphatase, permitted exchange studies with ureido- O^{18} -citrulline (109). Transfer of O^{18} to AMP indicated interaction between the ureido group and ATP. Absence of partial reactions demonstrable by O^{18} exchange was consistent with a concerted reaction requiring the simultaneous presence of both ATP and aspartate.

Although the role of acetylglutamate in the synthesis of carbamyl phosphate by animal enzymes remains obscure, Rajman & Grisolia (110) have observed that the cold inactivation of carbamyl phosphate synthetase from frog liver requires acetylglutamate—another example of the increased lability, under certain conditions, of enzymes in contact with substrates or cofactors.

Severina (111) has reinvestigated the earlier-debated possibility that

glutamine contributes to urea synthesis without release of free NH_3 ; in rat liver slices, urea synthesis from glutamine did not exceed that from NH_3 , while suppression of urea synthesis by omitting bicarbonate led to the accumulation of free NH_3 from glutamine.

Ornithine is an effective antidote for experimental hyperammonemia (112); a similar effect of arginine had been reported earlier by Greenstein *et al.* (113).

The recognition of argininosuccinic acidemia in man as a metabolic disorder associated with mental deficiency (114), and, in the cases studied, the higher concentration of argininosuccinate in cerebrospinal fluid than in blood, has drawn attention to the steps of urea synthesis in brain. In extension of earlier reports by Spron *et al.* (115) that urea could be made from arginine in brain, Tomlinson & Westall (116) have demonstrated argininosuccinase as well as argininosuccinate synthetase in rat brain. A more detailed examination of the subject by Ratner *et al.* (117) reports the presence of both argininosuccinase and the synthetase in the brains of several species including man. The synthetase activity of human brain appeared too low to account for the large accumulations of argininosuccinate reported in the disease, and it was therefore suggested (117) that a block in the splitting enzyme of extracerebral tissues, such as the kidney, might also be postulated. It is notable, however, that there was no decreased ability to form urea in the patients originally studied (118).

Metabolic regulation.—The repressive effect of arginine on its biosynthetic enzymes in bacteria is well-documented and is one of the early examples of end-product control of enzyme synthesis. Gorini (119, 120) has found that in *E. coli* strain B, in contrast, arginine stimulates the synthesis of at least two enzymes of the pathway: ornithine transcarbamylase and argininosuccinase. Although the relation between arginine action as a repressor and as a stimulator of enzyme synthesis is not clear, stimulation was interpreted as a reversal by arginine of the action of a more effective repressor not itself arising as a product of the biosynthetic pathway.

In further studies of the loss of arginine-glycine transaminidase activity from chick liver following dietary creatine, Walker (121) has examined some factors that influence restoration of enzyme activity after creatine withdrawal. Inhibitors of protein synthesis such as ethionine, or dietary deficiencies in protein or amino acids prevented enzyme restoration.

The increased excretion of renal creatine stimulated by vitamin E deficiency has been suggested by Fitch *et al.* (122) as the specific cause of reduced transaminidase levels in this condition. Coleman (123) has found that in mice with hereditary muscular dystrophy the enzyme level is reduced by much lower dietary creatine levels than in normal mice; high levels of glycine overcome the creatine-provoked reduction in enzyme level in both dystrophic mice and in normal mice, as observed also by Van Pilsum (124).

Miscellaneous.—Studies of Hartman & Zimmerman (125) suggest that

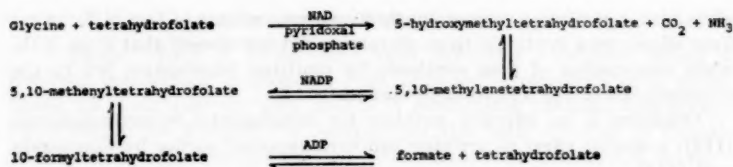


FIG. 1. Proposed reaction sequence in glycine fermentation.

the high arginine requirement for formation of the *Streptococcus faecalis* proteinase is explained by competition for the free amino acid by the arginine dihydrolase system. As with a number of analogous cases, peptide-bound arginine is used more efficiently.

Arginine is reported to protect rats from the convulsions provoked by increased O_2 tension (126). Since O_2 toxicity increases the ammonia content of brain and other tissues (126), the protective action of arginine can be rationalized as promoting the rate of urea formation.

GLYCINE AND SERINE

Glycine.—Further studies by Sagers & Gunsalus (127) of glycine fermentation by *Diplococcus glycinophilus*, which degrades glycine to ammonia, acetate, and CO_2 , are consistent with decomposition to formate and CO_2 through a tetrahydrofolate-dependent pathway (Fig. 1).

Acetate formation was accounted for by the alternative utilization of hydroxymethyl-tetrahydrofolate for serine formation from glycine and the subsequent deamination of serine to pyruvate; these reactions were both demonstrable in cell extracts. Another fermentative pathway of glycine has been studied by Stadtman (128) in extracts of *Clostridium sticklandii*, which convert glycine to acetate with the formation of ATP.

The synthesis of aminoacetone from glycine and pyruvate (or other Krebs cycle intermediates) in liver mitochondria (129) is probably another example of the pathway of aminoacetone formation, from acetyl CoA and glycine, found in *S. aureus* (130). The degradation of aminoacetone in liver preparations may go principally via deamination to methylglyoxal, since inhibitors of monoamine oxidase were observed to inhibit aminoacetone decomposition (129). These reactions suggest the possibility of a cyclic pathway resulting in complete glycine oxidation:



Evidence that, in *E. coli*, glyoxylate is not an intermediate in the conversion of glycine carbons to thymine is based on dilution experiments by Pitts *et al.* (131) with unlabeled glyoxylate. Since extracts transaminate glyoxylate (131), the absence of dilution via this reaction must be explained by the postulate that glyoxylate is degraded *in vivo* entirely through other pathways. Jenny & Leuthardt (132) have found that guinea pig liver slices label glutamine from glycine-2- C^{14} in a pattern consistent with the production of a labeled one-carbon fragment that recondenses with glycine to form serine and thence 2,3-labeled pyruvate. A more specialized degradative pathway for glycine in rabbit reticulocytes may be the glycine-succinate condensation suggested by Urbahn & Rapoport (133) from the observation that unlabeled δ -aminolevulinate, but not formate or oxalate, dilutes the $C^{14}O_2$ released from glycine-1- C^{14} .

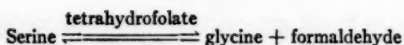
Hoskins & Mackenzie (134) have solubilized by sonic vibration the dehydrogenase portions of the mitochondrial oxidases which convert dimethylglycine to sarcosine and sarcosine to glycine—reactions concerned in the utilization of methyl groups. Phenazine methosulfate was active as a carrier for preparations that no longer reduced 2,6-dichlorophenol-indophenol.

Rogers *et al.* (135) found that the early initiation of growth of lactobacilli (and other bacterial strains) is stimulated by glucosylglycine, although glucose carbons were not incorporated in cells, nor was there other evidence for hydrolysis to glucose and glycine. The possibility that the compound participates in forming a required metabolite is further suggested by the isolation of *E. coli* mutants which were stimulated by glucosylglycine, or by other glycine peptides such as glycylasparagine (136). The more efficient use of glycylglycine than of glycine by glycine-requiring *E. coli* strains has been concluded by Levine & Simmonds (137) to be attributable to the more rapid uptake of the peptide.

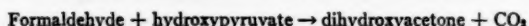
Serine.—Detailed reports by Ennor *et al.* (138, 139) describe the isolation from earthworms of D-serine, as the free compound, as serine guanidoethanol phosphate diester (lombricine), and as serine ethanolamine phosphate diester. The L-configuration of serine in the serine ethanolamine phosphate diester of reptilian muscle, however, originally reported by Roberts & Lowe (140), was confirmed (138); the compound is also found in birds, amphibia, and fish and can be formed by chicken kidney homogenates (141).

The O-sulfate ester of serine, chemically synthesized by Dodgson *et al.* (142), is desulfated by rat liver preparations; synthetic threonine- and hydroxyproline-O-sulfate esters are resistant to hydrolysis. Wilson *et al.* (143) have shown by isotope dilution experiments that neither free ethanolamine nor phosphoethanolamine is an intermediate in the formation of phospholipid ethanolamine from labeled serine in rat brain and liver preparations.

Serine-glycine interconversion.—A pathway of incorporation of the β -carbon of serine into dihydroxyacetone has been outlined by Batt *et al.* (144) as composed of reactions catalyzed by serine aldolase and transketolase:



6.



This was demonstrated by the incorporation of label from 3-C¹⁴-serine into triose—a reaction that is dependent on hydroxypyruvate and tetrahydrofolate in the presence of the purified enzymes.

Blakley (145) has further purified serine transhydroxymethylase from rabbit liver and provided spectral evidence for the reaction of glycine and N⁸,N¹⁰-methylene tetrahydrofolate to form tetrahydrofolate, and of serine and tetrahydrofolate to form methylene tetrahydrofolate.

SULFUR AMINO ACIDS

Methionine methyl synthesis.—Studies by several groups continue to focus on the individual reactions and cofactors in methionine synthesis from homocysteine and serine, a reaction known to have multiple requirements including a folic acid coenzyme, ATP, and a protein-bound form of vitamin B₁₂. The protein-cobalamin factor can be formed enzymatically in *E. coli* extracts on incubation with cobalamin, Mg⁺⁺, ATP, and NAD (146). Alternatively, the cobalamin factor may be purified directly from extracts of cells grown on vitamin B₁₂ (146, 147, 148). The protein factor, prepared by either method, is required for methionine synthesis when tetrahydrofolate replaces heated bacterial extract as the one-carbon carrier. It cannot be replaced (146, 147) by the dimethyl benzimidazole cobamide coenzyme of Barker (149).

A detailed paper from Buchanan's laboratory (150) described the partial purification from *E. coli* of several collaborative enzymes in the pathway of methyl group synthesis. The first of these, serine hydroxymethylase, transfers the one-carbon fragment from serine to tetrahydrofolate. Two additional separate protein fractions are then required for the reduction and transfer to homocysteine of the one-carbon moiety of the tetrahydrofolate coenzyme. One of these fractions is a cobalamin-protein, perhaps identical with that cited above. Cofactor requirements include ATP, a flavin (either flavin mononucleotide or FAD), and NADH₂. The required flavin, described in earlier preliminary reports, is regarded as a participant, together with reduced pyridine nucleotide, in reducing the transferred carbon from the hydroxymethyl to the methyl level. Except for the serine hydroxymethylase reactions, the precise nature of participation of the individual protein fractions and cofactors remains poorly defined.

The B₁₂-apoenzyme was purified by Takeyama *et al.* (151) from mutant cells of a methionine-B₁₂ mutant grown on methionine, and some features of its *in vitro* combination with cyanocobalamin were investigated. Tetrahydrofolate, FAD, ATP, and Mg⁺⁺ appeared to participate in its formation. Failure of the dimethyl-benzimidazole cobamide coenzyme to replace cyanocobalamin efficiently in the *in vitro* formation of the B₁₂ holoenzyme (151),

together with spectral and electrophoretic observations of the B₁₂-enzyme and the isolated protein-free cobamide (152), distinguished the latter from either cyanocobalamin or the dimethyl benzimidazole cobamide.

Some uncertainty remains concerning the nature of the endogenous folic coenzyme and the relation between this factor and the B₁₂ coenzyme requirement. Jones *et al.* (153) found that reduced pteroyl triglutamate as well as its N⁸- and N¹⁰-derivatives can replace tetrahydrofolate for methionine synthesis in the *E. coli* system. Unlike tetrahydrofolate, the triglutamates do not require added cobalamin, and in this respect as well as in their greater stability to air, they more closely resemble the endogenous cofactor activity of extracts of heated *E. coli*. A preliminary communication by Sakami & Uktins (154) describes the chemical synthesis of presumptive 5-methyl tetrahydrofolate, and the activity of this compound in donating methyl to acetylhomocysteine in a pig liver preparation. This report is consistent with the findings of Buchanan *et al.* (155, 156) who suggest that a N-methyl folate derivative, formed enzymatically from N⁸,N¹⁰-methylenetetrahydrofolate, can methylate homocysteine in the presence of the B₁₂ enzyme fraction.

Repression of methionine synthesis from serine and homocysteine, studied in detail by Rowbury & Woods (157), is effected by a number of methionine analogues, although in all cases less effectively than methionine. Support for cystathionine as a precursor of homocysteine in methionine biosynthesis is provided by the observation (158) that cystathionase of *E. coli*, as well as the homocysteine methylating system, is repressible by methionine.

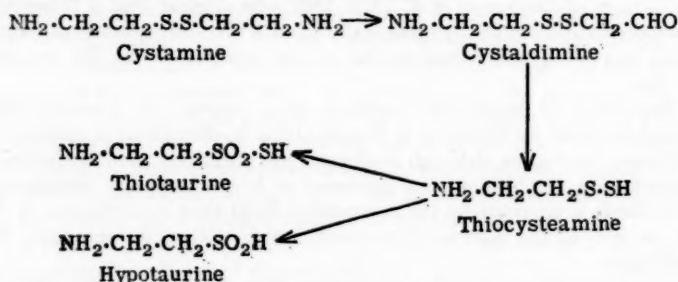
Transmethylation.—Further studies by Klee & Cantoni (159, 160) of thetin-homocysteine methyltransferase from horse liver suggest that the enzyme forms mixed disulfides with low molecular weight thiols, such as mercaptoethylamine and thioglycolate, producing distinct protein bands on modified cellulose columns. Homogeneity was restored by treatment with homocysteine followed by dialysis. Polymerization of the enzyme, presumably by intermolecular disulfide formation, yielded a constant relation of enzymatic activity to sedimentation constant over a considerable range of polymerization.

The distribution and specificity of another homocysteine methyl transferase of liver, the betaine-utilizing enzyme, were investigated in detail by Ericson (161, 162, 163). The enzyme, purified about 150-fold from pig liver, was essentially homogeneous with a molecular weight of 270,000 and contained no detectable B₁₂, folic, or pyridoxal coenzymes. The pig liver enzyme utilized dimethylacetothetin as well as betaine; however, the horse liver enzyme (164) utilized betaine as a methyl donor only relatively slowly, and thus differed from Ericson's transmethylation.

Greene & Davis (165) have reported the synthesis in jack bean root extracts of S-methylmethionine from methionine and S-adenosylmethio-

nine, the first example of the biosynthesis of a naturally occurring sulfonium compound other than S-adenosylmethionine. A second example is the formation of labeled dimethyl- β -propiothetin from labeled methionine in *Ulva lactuca* (166). Transmethylation to homocysteine from S-adenosylmethionine or S-methylmethionine has been observed by Turner & Shapiro (167) in extracts of seeds of several common plants.

Reactions of cysteine and related thiols.—A variety of degradative reactions involving cysteine or its relatives continue to be explored, principally in French and Italian laboratories. The decarboxylation product of cystine, cystamine, is converted to hypotaurine, and to the recently discovered thiotaurine, by a pig kidney preparation studied by Cavallini *et al.* (168). Since thiocysteamine, the persulfide analogue of cysteamine, is even more efficiently converted to the same products, it was regarded as an intermediate, the suggested reaction sequence being:



Another route of thiotaurine formation studied in the same laboratory (169) is transsulfuration from cysteine to hypotaurine, catalyzed by cystathionase. The enzymatic reactions appear to require preliminary oxidation in air of cysteine to cystine. Cystine is then the substrate for cystathionase cleavage to S-thiocysteine which can transfer a thiol to hypotaurine.

β -Sulfinylpyruvate, formed by oxidation of cysteine sulfinic acid either through deamination or transamination, is unstable, decomposing to sulfite and pyruvate. DeMarco & Coletta (170) have observed that, if cysteine or cystamine is present in such a reaction mixture, the sulfite liberated in the decomposition reaction may add to these compounds, perhaps nonenzymatically, to form S-sulfocysteine or S-sulfocysteamine.

Derivation of urinary thiosulfate from alanine thiosulfonate *in vivo*, described by DeMarco *et al.* (171), can be explained by transamination of the latter compound, as demonstrated in a rat liver mitochondrial preparation, to yield the corresponding keto acid which decomposes to pyruvate and thiosulfate (172).

Yolk sac or chick embryo is a source of several reactions studied in Fromageot's laboratory. A specific cysteic acid decarboxylase from chick

embryo liver fails to decarboxylate cysteine sulfuric acid which is, however, a competitive inhibitor of the reaction with cysteate (173). A partly purified protein fraction from yolk and yolk sac (174, 175) catalyzes several reactions of cysteine: (a) substitution of cysteine thiol by sulfite to form cysteic acid; (b) exchange of labeled H_2S with the sulfur of cysteine; (c) addition of a second cysteine to form lanthionine; or (d) hydrolysis, in the absence of sulfur compounds capable of replacing the cysteine thiol, to yield H_2S , NH_3 , and pyruvate. Evidence for a single enzyme was based on the constancy of the ratio for reactions 1 and 2 in different preparations, together with a scheme that would rationalize all four reactions as additions of water or sulfur compounds to a desulfhydrated enzyme-bound intermediate. The postulated enzyme, called cysteine lyase, is stimulated by pyridoxal phosphate. An analogous nonenzymatic sulfur exchange of cysteine with H_2S or sulfite is also catalyzed by pyridoxal phosphate and metal ions at $100^\circ C$ (176).

Fromageot & Patino-Bun (177) have described the polarographic measurement of sulfite production from cysteine sulfinic acid in oat-leaf extracts. The reaction was inhibited by maleate and stimulated by pyridoxal phosphate and oxalacetate or α -ketoglutarate; this is consistent with an initial transamination to β -sulfinylpyruvate which would rapidly decompose to yield sulfite.

An alliinase from onion, partly purified by Kupiecki & Virtanen (178), splits a number of alkyl cysteine sulfoxides, including alliin (the best substrate, although one not present in onions) to liberate pyruvate. Although only sixfold purified, the enzyme was stimulated by pyridoxal phosphate.

Other aspects of the synthesis of cysteine and methionine.—Several pathways of sulfur metabolism in the cockroach, *Blattella germanica*, involve intestinal symbionts (179, 180). Thus, S^{35} -cysteine is convertible to methionine only in xenic insects, but not in those reared without symbionts. Similarly, the reduction of sulfate to sulfite does not proceed in axenic insects. Evidence exists for the conversion in these insects of methionine to cysteine via homocysteine and the cystathionine pathway, and for oxidation of cysteine to pyruvate and sulfate via cysteine sulfinic acid and β -sulfinylpyruvate.

Further evidence of the utilization of selenium for the synthesis of selenomethionine and selenocysteine, which can be isolated from protein hydrolyzates, is based on studies with *E. coli* (181) and yeast (182).

Eagle *et al.* (183), investigating the paradox that cultured human cells can make cystine from methionine (probably through the cystathionine pathway) but yet require external cystine in addition to methionine, suggest that preformed cystine in the medium is necessary to prevent critical depletion of intracellular cystine by leakage. Other similarly explained external requirements include serine, inositol, pyruvate, and tryrosine (184).

Miscellaneous.—*Cis*- but not *trans*-crotylglycine acts as a competitive inhibitor of methionine for the growth of *E. coli* (185). Hence, the conformation of methionine necessary for relief of inhibition is identified as that cor-

responding to the *cis* isomer. The site of inhibition, whether at the level of membrane penetration or beyond, is unknown. Cysteine, at a high concentration relative to that of the substrate, is reported to inhibit glutamic-alanine transaminase in liver slices (186). Pyridoxal phosphate relieves the inhibition, so that a consistent mechanism might be the binding of pyridoxal as a thiazolidine, a basis suggested earlier by Du Vigneaud *et al.* (187) for penicillamine inhibition of pyridoxal phosphate reactions.

The toxic action of S-dichlorovinyl-L-cysteine in producing aplastic anemia in cattle or growth inhibition in *E. coli* is reported by Daniel & Schultze (188) to be rather specifically inhibited (in its growth-restraining effects on *E. coli*) by L-phenylalanine or L-tyrosine. The D-isomers were inactive, as were many other L-amino acids.

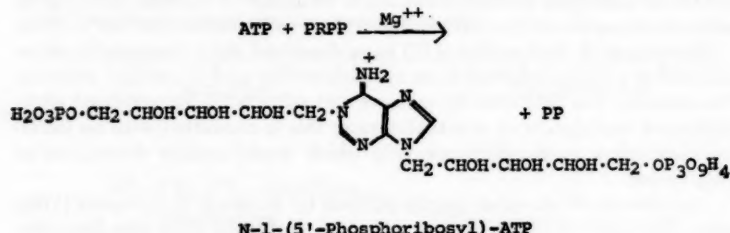


FIG. 2. First reaction of histidine biosynthesis.

Gorini (189) has described an effect of L-cysteine (or other disulfides or thiols at higher concentrations) in overcoming the protracted lag phase exhibited by coliform organisms grown in anaerobic media. The role of sulfur compounds in initiating anaerobic cell growth has no present explanation, but Gorini cites a possibly analogous need for dithiols in metazoan cell division noted by Mazia & Zimmerman (190).

Analysis of sulfur amino acids in human plasma under conditions which prevent cysteine oxidation indicated the normal postabsorptive levels of cystine and cysteine as about 1 mg per cent and 0.4 mg per cent, respectively (191). Frimpter (192) has isolated the mixed disulfide of L-cysteine and L-homocysteine from the pooled urine of cystinurics. This compound has not previously been found to occur naturally.

HISTIDINE

Biosynthesis.—The work of Ames *et al.* (193) defines the first reaction of histidine biosynthesis as the condensation of ATP and 5'-phosphoribosylpyrophosphate to form N-1-(5'-phosphoribosyl)-ATP and pyrophosphate (Fig. 2):

Phosphoribosyl ATP can be further converted to Compound III, a

compound earlier described by Moyed & Magasanik (194) as a precursor of imidazoleglycerol phosphate. Phosphoribosyl ATP may represent the accumulated but uncharacterized intermediate before Compound III, discovered by Klopotoski *et al.* (195). From the work of Ames *et al.* (193), $(\text{NH}_4)^+$, rather than glutamine, appears to be the preferred source of the N atom that completes the newly formed imidazole ring. As outlined earlier by Moyed & Magasanik (194), the cleavage and cyclization yield imidazoleglycerol phosphate and aminoimidazolecarboxamide ribotide.

The enzyme that catalyzes formation of phosphoribosyl ATP is missing from G-mutants of *Salmonella* (which can however convert phosphoribosyl ATP to imidazoleglycerol phosphate). Histidine represses the synthesis of this enzyme in a manner similar to the repression shown earlier for other histidine biosynthetic enzymes. The initial position of this step in histidine biosynthesis is also supported by direct inhibition of enzyme action by histidine. Although the general outline of imidazole-ring synthesis has been established in the past few years, the structure and role of Compound III and the mechanism of the ring cleavage, nitrogen insertion, and cyclization reactions will require much clarification.

Moyed (196) reports that feed-back inhibition of one of the earlier histidine biosynthetic steps by 2-thiazolealanine accounts for the inhibiting action of this compound on histidine formation. Independence of the phenomena of product inhibition and product repression was indicated by isolating mutants which were resistant to feed-back inhibition both by the analogue and by histidine, but which remained sensitive to the repressive action of histidine.

The recently observed phenomenon of enzyme complementation, obtained by mixing extracts of intragenic mutants, is exemplified by Loper's report (197) that imidazoleglycerol phosphate dehydrase activity appears on mixing extracts derived from individual *Salmonella* mutants blocked at this locus.

Purification of histidinol dehydrogenase, essentially to homogeneity, by exploiting the derepressive technique in *Salmonella* mutants, has been reported in a preliminary communication by Loper & Adams (198).

Degradation.—An analysis by Peterkofsky (199) of the histidase reaction, which deaminates histidine to urocanate, is consistent with the participation of an amino enzyme intermediate. Urocanase has been extensively purified from beef liver by Rao & Greenberg (200) and the product, by spectral comparison with related compounds, found consistent with the postulated 4(5)-imidazolone-(5)4-propionic acid. This work confirms earlier reports from the same laboratory (201) and by Brown & Kies (202). Gupta & Robinson (203) have prepared a highly purified urocanase having a pyridoxal phosphate requirement.

Further reactions of imidazoline propionate are either spontaneous decomposition to formylisoglutamine or enzymatic cleavage of the ring to

formiminoglutamate. Imidazolone propionate hydrolase, catalyzing the latter reaction, has been partly purified both from *Pseudomonas* by Rao & Greenberg (204) and more extensively from rat liver by Snyder *et al.* (205). In studies with the rat liver enzyme (205), the substrate was protected from air in order to minimize the competing spontaneous reaction. Under these conditions formiminoglutamate, the enzymatic product, accounted quantitatively for the utilization of imidazolone propionate.

A detailed study by Tristram (206) of the utilization of L- and D-histidine by *Paracolobactrum aerogenoides* indicates that L-histidine is degraded through the urocanate-glutamate pathway now established for other bacteria and for liver, but suggests a different pathway for D-histidine. The possibility of racemization was admittedly not eliminated.

A distinction has been made between histidine-decarboxylating enzymes from various tissues. The enzyme in kidney cortex was earlier identified as a decarboxylase for other aromatic compounds (such as DOPA) as well, and has a considerably higher K_m for histidine than the histidine decarboxylase of mast cells or fetal tissues (207, 208). Unlike the mast-cell enzyme, the kidney decarboxylase is inhibited by α -methyl DOPA and 5-hydroxytryptamine. Inhibition by the former compound *in vivo* [Werle (209)] supports the belief that the kidney enzyme is not physiologically significant as a source of histamine, since effective doses of inhibitor produced no decrease in the urinary excretion of histamine. Conversely, Mackay *et al.* found that the high level of histidine decarboxylase in rat hepatoma, associated with high urinary excretion of histamine, was not accompanied by an increase in DOPA decarboxylase (210).

Miscellaneous.—Thiolurocanic acid has been identified as a product of ergothioneine degradation in extracts of *Alcaligenes faecalis* (211). Uncertainty remains concerning the identity of this enzyme and urocanase. Two reports indicate that liver disease in humans may lead to the accumulation and excretion of urocanate: urocanate has been isolated from the urine of a patient with hepatic coma (212) and has also been measured at excretion rates of 10 to 80 mg per 12 hours in the urine of subjects with kwashiorkor (213). Urocanase has been reported present in guinea pig skin (214); appreciable concentrations of urocanate in skin had been noted earlier. The greatly elevated levels of blood and urine histidine in two siblings (215) may portend the definition of another human genetic defect in amino acid metabolism.

LYSINE

Biosynthesis.—The work of Gilvarg and associates has now largely outlined the formation of diaminopimelate in *E. coli*, and hence, the establishment of this pathway for lysine biosynthesis. Full descriptions have appeared of some of the enzymes and intermediates in the later steps of diaminopimelate formation: N-succinyl-L- α -amino- ϵ -keto pimelate (216), the transaminase (217) which converts this keto pimelate to the corresponding succinylated

L-diamino compound, and the deacylase (218) which removes the succinyl group. A further necessary step in lysine biosynthesis is the racemization of L- α - ϵ -diaminopimelate, described earlier by Antia *et al.* (219), to the meso-isomer, the requisite substrate for decarboxylation. The condensation of a 4-carbon and 3-carbon unit to form the carbon skeleton of diaminopimelate, suggested in earlier work of Gilvarg (220), has been further clarified by evidence that aspartic semialdehyde may be the specific intermediate form of the 4-carbon portion (221). The belief that the diaminopimelate pathway represents the only significant biosynthetic route to lysine in *E. coli* is supported by the work of Rhuland & Hamilton (222) who found that γ -methyl-diaminopimelate can replace diaminopimelate, but not lysine, in a lysine auxotroph. Utilization of diaminopimelate for lysine synthesis in higher plants is indicated by Finlayson & McConnell in isotope experiments with wheat (223).

Sagisaka & Shimura (224) report that the yeast enzyme which reduces α -amino adipate to adipic- δ -semialdehyde catalyzes a substrate-dependent ATP-pyrophosphate exchange, consistent with the postulated δ -adenyl- α -amino-adipate as an intermediate. Mattoon *et al.* (225) have isolated from lysine-requiring yeast mutants a compound with certain of the properties expected for this proposed intermediate. In intact yeast cells, labeled α -amino-adipate, while extensively converted to L-lysine, also gives rise to two minor labeled products, δ -aminovalerate and α -hydroxy- ϵ -aminohexanoate both rather directly derivable from the substrate by plausible reactions (226).

Degradation.—In *Pseudomonas*, an initial step in lysine degradation is oxidative decarboxylation to δ -aminovalerate (227). Recent studies of lysine oxidase by Itada *et al.* (228) indicate an oxygenase mechanism, since atmospheric O_2 is the source of about 0.5 atoms in the carboxyl of δ -aminovalerate; the failure to observe incorporation of at least a full atom was attributed to exchange during isolation. A subsequent reaction in this pathway, studied by Ichihara & Ichihara (229), is catalyzed by a rather specific dehydrogenase that converts glutaric semialdehyde to glutarate.

The position of pipercolic acid as a mainstream degradative intermediate in vertebrates is not unequivocally established, although the conversion of L-lysine to pipercolate has been observed in animals, plants, and molds. Recent studies by Rothstein & Greenberg (230) have documented the conversion of labeled pipercolate to α -amino adipate and glutarate. Boulanger & Osteux (231), however, report that labeled pipercolate was not significantly degraded either in the intact rat or in rat liver homogenates, nor did carrier α -amino-adipate become labeled. These directly conflicting observations remain unresolved.

Miscellaneous.—Since *trans*, but not *cis* 4-5 dehydrolysine is an effective antagonist of lysine for the growth of lactobacilli, the *trans* conformation is suggested by Davis *et al.* (232) as requisite for the biological utilization of lysine. As in the case of methionine conformation, similarly investigated in

the same laboratory (185), the step in biological utilization at which the amino acid is antagonized is not defined. Somerson *et al.* have found that α -aminoadipate reverses the inhibition of penicillin formation by lysine (233), an effect which may be explained by the possible status of δ -(α -amino-adipyl) cysteinyl-valine as a penicillin precursor—suggested by the earlier work of Arnstein & Morris (234).

Hydroxylysine-lysine interrelations in *Lactobacillus mesenteroides* have been studied by Smith *et al.* (235). Cells lyse readily after exhaustion of limiting lysine, but are stabilized by the addition of δ -hydroxylysine, which is incorporated into cell walls; increased cell stabilization by hydroxylysine was therefore suggested as the means whereby hydroxylysine appeared to spare the lysine requirement for growth. Interconversion of lysine and hydroxylysine was ruled out by isotope experiments.

PROLINE

Subsequent to his earlier studies of the interconversion of proline and glutamate, Strecker (236) now describes a NAD dehydrogenase, partly purified from beef liver, which oxidizes Δ^1 -pyrroline-5-carboxylate to glutamate. The substrate used was the chemically synthesized racemate earlier purified by Strecker (237); an unexpected finding was the apparent conversion of DL- Δ^1 -pyrroline-5-carboxylate to D- and L-glutamate at equal rates. A brief publication by Wiame *et al.* (238) also notes the existence of a similar reaction, specific for NADP, in extracts of *Bacillus subtilis*.

HYDROXYPROLINE

Distribution.—Two reports document the occurrence of hydroxyproline in the protein of plant cell walls (239, 240). A detailed study by Radhakrishnan *et al.* (241) of the seasonal and anatomic distribution of L-allohydroxyproline in *Santalum album* shows it to be present in all parts of the tree, and attaining levels of 10 per cent of the dry weight of the fruit pericarp. The difficulty in obtaining biosynthetically labeled hydroxyproline, because of its presumptive absence from microorganisms in general, lends particular interest to its reported occurrence in hydrolyzed ethanol extracts of *Chlorella* (242, 243). Detailed authentication of the isolated compound is awaited.

Collagen biosynthesis.—No definitive picture is yet at hand of reactions in hydroxyproline synthesis, or their relation to collagen formation or ascorbate requirements. In several earlier reports by Robertson *et al.* (244) and Green & Lowther (245), collagen-forming preparations were found to incorporate labeled hydroxyproline from labeled proline with a ratio of specific activity for collagen hydroxyproline/proline greater than one. Since such ratios exclude certain models for the hydroxylation of precursor peptide-bound proline, it is of interest that in a careful study of labeled insoluble collagen from guinea pig skin, Hausmann & Neuman (246) report specific

activity ratios distributed randomly around unity for samples obtained between 3 and 24 hours after administering labeled proline.

Ascorbate added *in vitro* was found by Van Robertson & Hewitt (247) to stimulate the incorporation of C^{14} proline as hydroxyproline in suspended tissue *brevi* obtained from the carrageenin granulomas of scorbutic (but not of normal) guinea pigs.

After a single dose of labeled proline, the isotope content in peptide hydroxyproline of rat urine fell at a rate consistent with several collagen pools of different turnover rates (248). Both these observations and others (249), showing a rather constant rate of urinary excretion of peptide-bound hydroxyproline, increased by ingesting gelatin, support the idea that the urinary hydroxyproline reflects collagen degradation. Consistent with this interpretation is the relatively large excretion of hydroxyproline in young rats or humans (248, 250) and the report by Martin *et al.* (251) that the scorbutic guinea pig excretes less and the lathyritic guinea pig more hydroxyproline than control animals. It would be of interest to measure urinary hydroxy-

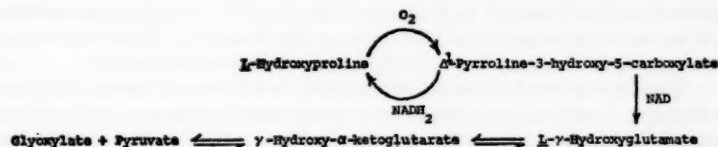


FIG. 3. Reactions in the mammalian metabolism of L-hydroxyproline.

proline excretion in the insulin-treated animal, in which elevated tissue levels of hydroxyproline have been observed (252).

Degradative metabolism.—Adams & Goldstone (253) have described the large-scale preparation and detailed characterization of Δ^1 -pyrroline-3-hydroxy-5-carboxylate, an initial oxidation product of L-hydroxyproline in mammalian tissues. Availability of the pyrroline product permitted the definition of two further reactions in liver preparations: NADH_2 -linked reduction of the pyrroline compound back to L-hydroxyproline (254), and NAD-linked oxidation to L- γ -hydroxyglutamate (255). Enzymatically formed γ -hydroxyglutamate, isolated as an enzyme product and hence documented as a mammalian metabolite, was found to represent the isomer expected from the configuration of L-hydroxyproline, thus providing independent confirmation of the earlier assignment of configuration to the four isomers as obtained synthetically by Benoiton *et al.* (256).

The further metabolism of L- γ -hydroxyglutamate involves initial transamination to the corresponding α -keto acid (257, 258) and cleavage of the keto acid to glyoxylate and pyruvate (257). These reactions complete a sequence from L-hydroxyproline to familiar metabolites (Fig. 3).

The earlier suggestion of Dekker that γ -hydroxyglutamate forms glyoxylate and alanine by direct cleavage (259) has been withdrawn (260) in favor of support for the transamination-cleavage sequence above. Since pyruvate is active as an amino acceptor in the transaminase reaction (258), it could function catalytically in coupling transamination to a subsequent cleavage reaction and would explain the earlier observed (259) conversion of γ -hydroxyglutamate to glyoxylate and alanine.

PHENYLALANINE AND TYROSINE

Biosynthesis.—Some of the early reactions in aromatic biosynthesis, initially defined in bacteria, have been described in higher plants and indicate the same prearomatic pathway established with bacterial mutants, as reviewed by Davis (261) and Sprinson (262). Thus, Nandy & Ganguli (263) report phosphoenolpyruvate and erythrose-4-phosphate as optimal substrates for 5-dehydroshikimate formation in extracts of mung-bean seedlings; 5-dehydroshikimate reductase has also been partly purified from the same source (264). Extensive purification of 5-dehydroshikimate reductase from pea epicotyls is described by Balinsky & Davies (265), who also present data on the structural requirements for substrate binding (266). The same authors have purified dehydroquinase eightfold from cauliflower buds (267).

The observations by Doy & Gibson (268), that during nitrogen starvation wild-type *Aerobacter aerogenes* or tryptophan auxotrophs accumulate large quantities of phenylpyruvate, *p*-hydroxyphenylpyruvate, and *p*-hydroxybenzaldehyde, are consistent with the known status of the aromatic keto acids as immediate precursors of the corresponding amino acids. Tryptophan auxotrophs were found to accumulate these compounds faster than wild-type cells, a fact attributed to the existence of a common non-nitrogenous precursor of tryptophan, phenylalanine, and tyrosine, and to the plausibly greater derepressed activity of earlier prearomatic enzymes in the tryptophan-auxotroph than in the wild type. Certain *A. aerogenes* mutants blocked between the last common aromatic precursor and anthranilate accumulate a number of phenolic and diphenolic compounds (269) whose exact origin remains obscure.

Phenylalanine.—Studies by Kaufman (270) of the oxidized form of the pteridine which participates in phenylalanine hydroxylation indicate this as the 5,6-dihydropteridine. It can be formed enzymatically or by chemical oxidation with 2,6-dichlorophenol-indophenol. Identification was based on elimination of the two other possible dihydropteridines.

Mice with "dilute" skin color showed a reduction of phenylalanine hydroxylase activity in liver homogenates (271). However, the reduction of activity could not be explained by a lowered concentration of enzyme in the supernatant fractions, but by the greater inhibitory activity of the particulate fraction.

Appreciable conversion of C^{14} -phenylalanine to C^{14} -benzoate, obtained

as urinary hippurate, was observed by Grümer in phenylketonuric humans, in whom blockage of hydroxylation might be expected to exaggerate minor alternate pathways (272). Koukol & Conn (273) have purified, from barley stems, a specific phenylalanase which catalyzes deamination to cinnamic acid.

Tyrosine.—Tyrosine-glutamate transaminase has been extensively purified from yeast in which tyrosine is metabolized through the transamination step and then to tyrosol (274). Urinary *p*-hydroxyphenylacetate can arise from at least two distinct routes of tyrosine degradation: from the oxidative decarboxylation of *p*-hydroxyphenylpyruvate, and from the oxidation of tyramine derived by tyrosine decarboxylation. An evaluation by Williams & Babuscio (275) of the relative magnitude of each pathway in the rat, using monoamine oxidase inhibitors, indicates *p*-hydroxyphenylpyruvate as the predominant source.

The low activity of *p*-hydroxyphenylpyruvic oxidase in the livers of fetal and new-born animals is attributed, at least in part, by Knox & Goswami (276, (277) to the higher proportion of inactive enzyme, activatable by reducing agents such as ascorbate, glutathione, or 2,6-dichlorophenol-indophenol. Fetal enzyme behaves, therefore, like the oxidase of adult animals, being inactivated as the result of a tyrosine load and requiring reducing agents for restoration of activity (276). In approximately 1 per cent of infants, high urinary excretion of *p*-hydroxyphenylpyruvate, *p*-hydroxyphenyllactate, and tyrosine has been observed even on a normal diet and with no evidence of ascorbate depletion (278); after a time the hydroxyphenyluria ceased abruptly without treatment. One explanation considered was simply a wide distribution curve for the time of appearance of the tyrosine-degrading enzymes. Seegmiller *et al.* (279) have described a sensitive and specific enzymatic method for detecting homogentisate in biological fluids; none was detected in normal human plasma, while alkaptonuric plasma contained appreciable quantities and showed large increases after tyrosine ingestion.

The requirement both for molecular oxygen and for Cu^{++} in tyrosine iodination by rat thyroid can be replaced by hydrogen peroxide (280). Desulfation of S^{35} -tyrosine sulfate in the intact rat is negligible (281); the finding in urine of S^{35} -*p*-hydroxyphenylacetate-O-sulfate and another unidentified sulfur-containing metabolite suggests that oxidation of tyrosine-O-sulfate proceeds through a pathway parallel to that of nonsulfated tyrosine.

Catecholamines.—Murphy & Sourkes have extended observations on the *in vivo* action of α -methyl-*m*-tyrosine as an inhibitor of the decarboxylation of DOPA (282): D- as well as L-DOPA gave rise to urinary DOPAmine, both reactions being inhibitable *in vivo*. DL-*m*-Tyrosine also led to the excretion of excess DOPAmine in the rat, although the order of hydroxylation and decarboxylation in the conversion is unknown (283). The low levels of

epinephrine in the plasma of phenylketonurics is perhaps explainable by the inhibiting action of phenylpyruvate on epinephrine synthesis from tyrosine, demonstrated in guinea pig slices by Boylen & Quastel (284). Accumulation of DOPA in the inhibited slices suggests DOPA decarboxylase as the specific site of inhibition.

Tyrosinase.—Experiments by Dressler & Dawson with Cu^{64} show that purified mushroom tyrosinase does not exchange its functional copper in the absence of substrate (285), while functioning enzyme does incorporate radioactive cupric ions (286). Maximal exchange of Cu was observed with high catecholase preparations during the oxidation of *o*-diphenols rather than monophenols. From the difference in Cu exchange with the two types of substrate, the authors suggest that monophenol and *o*-diphenol sites are distinct, and that the corresponding *o*-diphenols may not be intermediates in the oxidation of monophenols.

The lag before initiation of monophenol oxidation with relatively crude tyrosinase preparations has been attributed by Karkhanis & Frieden to the action of an inhibiting protein, separable from tyrosinase on purification (287). Since Cu-free apotyrosinase is also an inhibitor, and since incubation with Cu^{++} converts the inhibitory protein to an enzymatically active one, Karkhanis & Frieden suggest that apotyrosinase is itself the endogenous inhibitor responsible for the lag (288).

The marked lag in oxidizing tyrosine exhibited by solubilized, partly purified tyrosinase from hamster melanoma is reduced by DOPA or ascorbate, or by increasing the amount of enzyme (289). Similar observations had been reported earlier (290) but only in homogenates, in which the enzyme is particulate.

Data of Bu'Lock (291) based on absorption spectra, fluorescence, and chemical reduction products implicate a transient intermediate between 5,6-dihydroxyindole and melanin, perhaps the dimer, 5,6-dihydroxyindolyl-indole-5,6-quinone. Some properties of a microsomal tyrosinase from pupal stages of *Drosophila* have been described (292); an interesting feature is inhibition by low concentrations of chloramphenicol. Increased tyrosinase activity in goldfish skin accompanies the darkening induced by salt addition to the medium (293). ACTH also induces melanogenesis when added *in vitro* to cultured goldfish tail.

Phenylketonuria.—Production of certain clinical features of phenylketonuria by feeding high-phenylalanine diets has been observed in monkeys and rabbits. Waisman *et al.* (294) report that infant macaques receiving a high daily ration of phenylalanine showed reduced learning capacity and convulsions. Neurological disturbances and lesions were reported by Ammon (295) in young rabbits treated pre- and postnatally with DL-phenylalanine. Attempts to alleviate the disease in humans by supplemental doses of glutamine and asparagine, used as amino donors in order to lower keto acid levels, did succeed in reducing urinary phenylpyruvate-to-phenylalanine ratios in

two subjects; a slight improvement in mental performance was also reported in two of three patients (296).

TRYPTOPHAN

Synthesis.—Evidence for an intermediate between anthranilate and anthranilic deoxyribulotide has been obtained by Doy *et al.* (297). The postulated structure, N-(5'-phosphoribosyl) anthranilic acid, was originally proposed by Yanofsky (298) as the expected condensation product of 5'-phosphoribosyl pyrophosphate and anthranilic acid; by rearrangement it could yield the deoxyribulotide as the first stable intermediate after anthranilate. Doy *et al.* (297) observed a transient fall in fluorescence in bacterial extracts on mixing anthranilate and 5'-phosphoribosyl pyrophosphate. Fluorescence was restored in extracts of mutants, blocked in the formation of the deoxyribulotide, as the intermediate spontaneously decom-

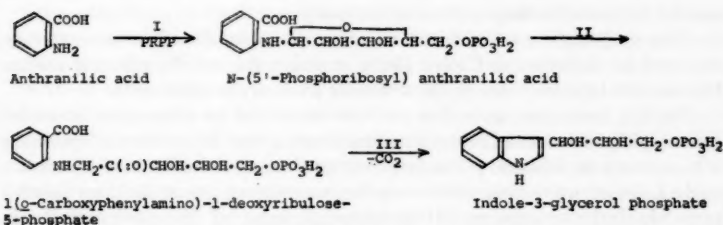


FIG. 4. Intermediates between anthranilic acid and indoleglycerol phosphate.

posed to anthranilate; the complementing action of mutant extracts suggested that the unstable compound could be converted enzymatically to indoleglycerol phosphate. The rearrangement reaction in step II (Fig. 3) is enzymatic, since mutants exist whose behavior corresponds to the absence of steps I, II, or III, respectively (297).

By depressive growth conditions, Gibson & Yanofsky (299) have increased the level of the enzyme that catalyzes step III, indole-3-glycerol phosphate synthetase, some sixtyfold over that of wild-type extracts, and have purified it seventeenfold further by fractionation; no evidence for more than one enzyme in this complex step was obtained by purification, or by mixing extract fractions or mutant extracts.

Genetic and physiologic aspects of the next step, that of tryptophan synthetase, continue to be pursued by several groups. The stimulation of tryptophan synthetase formation by anthranilate or 3-methyl tryptophan is interpreted by Lester & Yanofsky (300) as resulting from the inhibition of endogenous tryptophan synthesis. A protein antigenically related to tryptophan synthetase from mutant *Neurospora* was found by Mohler & Suskind

(301) to show similar purification and sedimentation properties. Tryptophan-independent strains of *Neurospora* produce proteins with tryptophan synthetase activity, but differing in stability on dialysis (302). Further identification has been made of glyceraldehyde-3-phosphate as the triosephosphate that arises from the tryptophan synthetase reaction (303). Crude extracts of Bengal gram represent another plant source of the synthetase reaction (304).

Degradation.—7,8-Dihydroxykynurenine acid has been reported as a product of kynurenine acid in the quinoline pathway of tryptophan degradation in *Pseudomonas*, studied in Hayaishi's laboratory (305); it was earlier shown, both by Behrman & Tanaka (306) and Hayaishi *et al.* (307), to replace kynurenate in the formation of further intermediates in this pathway. A full report by Hayaishi *et al.* on the enzymatic formation from kynurenine acid of L-glutamate, D- and L-alanine, and acetate in the *Pseudomonas* system has also appeared (308). The considerable dilution of glutamate derived from C^{14} kynurenate suggests the resynthesis of glutamate from smaller degradative fragments of kynurenate.

The enzymatic racemization of tryptophan in *Pseudomonas* extracts, reported by Behrman & Cullen (309), explains the equally efficient utilization of both isomers through the aromatic pathway in these cells.

To the numerous quinoline derivatives found in urine can be added quinaldylglycyltaurine (310), identified in cat urine. Administered quinaldic acid appears as urinary quinaldylglycyltaurine, as does ingested kynurenine acid. Labeled quinolinic acid in urine was shown to arise from labeled keto-3-hydroxykynurenine (311), although most of the radioactivity in urine in these experiments represented unidentified metabolites.

Extensive purification of 3-hydroxyanthranilic oxidase is reported by Iaccarino *et al.* (312). The enzyme was stabilized by Fe^{++} , by the exclusion of O_2 , and by the presence of 3-hydroxyanthranilate; a prosthetic group was not identified. Ehrensward (313) has observed that the oxidation of 3-hydroxyanthranilate by human serum is partly inhibited by cyanide; small differences were noted in the extent of cyanide inhibition in sera from normal and schizophrenic subjects.

Tested as a possible intermediate by Tsubokura *et al.* (314), *o*-formamino-benzoylacetate was decomposed to anthranilate by dried cells which inducibly degrade indoleacetate to anthranilate. Interruption of the conversion of 3-hydroxykynurenate to 3-hydroxyanthranilate is postulated to explain the elevated excretion of 3-hydroxykynurenate and derivatives by mice with experimental hepatitis (315).

Alkaloid biosynthesis.—Tryptophan-3- C^{14} was converted to tropic acid in *Datura* (316). Exclusive labeling of the carboxyl of tropate is consistent with opening of the pyrrole ring and loss of the ring-N and the terminal two carbons of tryptophan in the process. Leete (317) has provided similar evidence for incorporation of tryptophan-2- C^{14} as the indole fragment of the *Rauwolfia* alkaloid, ajmaline. The incorporation of tritium from indole into one of the ergot alkaloids has also been reported (318).

Tryptophan pyrrolase.—Further studies of liver tryptophan pyrrolase by Feigelson & Greengard suggest that the activating microsomal factor is a heme compound (319). Enzyme stabilization by tryptophan *in vitro*, as well as the initial phase of increased enzyme activity following tryptophan *in vivo*, may be effected through this activation mechanism (320). The enzyme, which has been purified 300-fold from rat liver, is then inactive without added hematin (321). Pitot & Cho report an apparent release of soluble enzymes from microsomes as an energy-dependent process requiring ATP (322); the indication that the soluble enzyme may be activated by a microsomal heme fraction (319, 320) complicates these observations. Inhibition of tryptophan pyrrolase *in vitro* by 5-hydroxytryptamine or epinephrine (323) provides an additional possible regulating mechanism for enzyme activity. Tryptophan pyrrolase is one of several enzymes that are absent from primary liver hepatomes, either transplanted or chemically induced (324).

Hydroxylation and decarboxylation.—Hydroxylation to 5-hydroxytryptophan, of interest as the first step in 5-hydroxytryptamine formation, has been demonstrated *in vitro* by two groups: Freedland *et al.* (325) present provisional evidence for the reaction in rat liver homogenates, and find that NAD is a requirement; a more detailed report by Cooper & Melcer (326) describes the reaction in a particulate fraction of rat or guinea pig intestinal mucosal extracts. Curiously, the reaction proceeded anaerobically as well as aerobically, and was inhibited by the soluble fraction of homogenates. Cu^{++} was required, together with ascorbate or related compounds. Although decarboxylation of 5-hydroxytryptophan is the reaction of major physiological and pharmacological interest, an alternative pathway, involving transamination to 5-hydroxyindolylpyruvate and reduction to 5-hydroxyindolylactate, has been encountered by Spencer & Zamcheck (327). Axelrod & Weissbach (328) have purified a rather specific transferase which methylates N-acetyl-5-hydroxytryptamine from S-adenosylmethionine to form melatonin: of a number of tissues examined, the enzyme was found only in pineal gland.

Erspamer *et al.* (329) report that a variety of tryptophan analogues, including 4-hydroxytryptophan, N-acetyl-5-hydroxytryptophan, and N-acetyl-5-acetoxytryptophan, can be decarboxylated in tissue homogenates. However, when the N-acetyl compounds were fed to rats, it appeared from the excretion products that hydrolysis precedes decarboxylation (330). 4-Hydroxytryptophan, a possible precursor of psilocybin in the mushroom, is metabolized by rats (331) or man (332) to yield 4-hydroxytryptamine and 4-hydroxyindoleacetate. Doses up to 1 g by mouth had no overt pharmacological effects in human subjects (332).

Miscellaneous.—The inhibition by tryptophan of the growth of *Neurospora* wild type or mutants, and its relief by a number of amino acids, have been noted by Soboren & Nyc (333) but not yet explained. A detailed comparison by Benassi *et al.* (334) of several urinary tryptophan metabolites after a loading dose in human subjects suggests that considerably higher

levels of these compounds may be excreted by ambulatory schizophrenic subjects than by controls. L-Tryptophan is specifically and sensitively assayed by measuring the indole produced after treatment with *E. coli* tryptophanase (335).

LEUCINE, ISOLEUCINE, AND VALINE

Synthesis.—The probable first step in isoleucine biosynthesis, condensation of α -ketobutyrate and an acetal fragment from pyruvate to form α -aceto- α -hydroxybutyrate, has been studied in *E. coli* extracts by Leavitt & Umbarger (336). Several lines of evidence confirm the earlier expectation that the same enzyme also catalyzes α -acetolactate formation from pyruvate. Armstrong & Wagner (337) have purified from *Salmonella* extracts the reductoisomerase which catalyzes the conversion of the α -hydroxy- α -alkyl precursors of valine and isoleucine to the isomerized α - β -dihydroxy compounds. The enzyme requires Mg^{++} and $NADPH_2$; a B_{12} coenzyme has not been implicated. The simultaneous presence of a reductoisomerase and reductase remains unexplained. It has been suggested that the reductase may be an incomplete derivative of the reductoisomerase (337).

The next step in this biosynthetic sequence is the dehydration of the branched chain α - β -dihydroxy acids to form the immediate α -keto precursors of the amino acids. Dihydroxy acid dehydrase of *E. coli*, studied by Myers (338), catalyzes analogous reactions with the precursors of both valine and isoleucine. Examination by Wixom *et al.* of a number of vertebrates, microorganisms, and plants indicates a good correlation between the presence of the enzyme and nutritional independence of exogenous valine (339, 340).

In leucine biosynthesis, β -hydroxy- β -carboxy-isocaproate, the intermediate originally proposed by Strassman *et al.* (341) from labeling experiments, has been supported by Jungwirth *et al.* in direct experiments with a series of *Salmonella* mutants (342). The compound was isolated from a mutant blocked in a succeeding reaction; its almost exclusive formation from labeled α -ketoiso-valerate has also been shown.

Metabolic abnormalities.—The probable block in branched-chain amino acid metabolism in maple-sugar urine disease was demonstrable by Dancis *et al.* (343) in a diagnostically useful *in vitro* procedure in which leukocytes from the peripheral blood of infants showed little capacity to produce $C^{14}O_2$ from labeled valine, leucine, and isoleucine. The hypoglycemia, as yet obscure, induced by leucine in certain individuals, has been recently reviewed by DiGeorge & Auerbach (344). Studies of Johnson *et al.* (345) failed to demonstrate any peripheral effects of leucine on the glucose uptake of rat diaphragm. Fajans *et al.* (346) showed that increased sensitivity to leucine appears under conditions, such as chlorpropamide treatment, that stimulate islet-cell activity. These and earlier observations suggest that insulin release rather than insulin action is primarily affected by leucine or a derived metabolite.

Miscellaneous.—Sanwal & Zink (347) present evidence for the existence in *Bacillus subtilis* of an NAD-linked L-leucine dehydrogenase. The enzyme, purified about 26-fold, failed to catalyze L-alanine dehydrogenation, so that coupled transdeamination between leucine-alanine transamination and alanine dehydrogenase seems unlikely as an alternate pathway for the observed reaction. A detailed investigation by Haughton & King (348) of the induced formation of leucine decarboxylase in *Proteus vulgaris* reports rather wide but not identical structural requirements both for substrate and inducer activity. In the same organism, another pathway of leucine degradation, studied by Sasaki (349), involves decarboxylation of α -ketoisocaproate to isovaleraldehyde, a possible substrate for the formation of isovaleryl CoA.

Isotopic evidence of Butler & Butler (350) supports the formation, from valine and isoleucine, respectively, of the branched cyanogenetic glycosides of clover: linamarin and lotaustralin. These compounds are of economic interest because of their toxicity for cattle. Fowler *et al.* (351) report the isolation from mycobacterial culture filtrates of the N-acetyl forms of L-leucine and L-isoleucine.

MISCELLANEOUS

An interesting correlation of slow growth in certain *Salmonella* strains with increased oxidative dissimilation of amino acids and reduced incorporation of C^{14} from labeled acetate was noted by Stokes & Bayne (352). The over-all pattern of oxidative utilization of amino acids by *Salmonella* has also been described (353). Kirsten *et al.* (354) describe selectively decreased levels of a number of glucogenic amino acids in the livers of alloxan-diabetic rats, the decreases roughly paralleling those of oxaloacetate and pyruvate. Free amino nitrogen, although unchanged in the plasma of uremic subjects studied by Frimpter *et al.* (355), was increased by acid hydrolysis. High levels of phenacetylglutamine, together with unidentified hydrolyzable compounds, accounted for the increase.

α - γ -Diaminobutyric acid, previously described as a trace component of plants (356), has been isolated from *Lathyrus latifolius* by Ressler *et al.* (357) and identified as the chief toxic component. The lathyritic factor in *Lathyrus* species had been earlier recognized as β -amino-propionitrile, present naturally as the N- γ -glutamyl peptide. β -Aminoisobutyrate, recently described in the iris bulb (358), originates from thymine in this source (359)—probably through the pathway originally outlined by Fink *et al.* (360, 361) for the rat. *L. arabinosus*, which can utilize a number of N-benzoyl- and other N-acylamino acids, yields extracts capable of hydrolyzing these amino acid substitutes; good correspondence was noted between nutritional activity and substrate activity for deacylation (362).

Carnitine.—The biosynthetic origin of carnitine remains obscure. C^{14} , introduced as carnitine by Lindstedt & Lindstedt (363), is retained in the intact rat for long periods of time, with only a small proportion being lost

within 10 days either as CO_2 or in the urine. Similar findings by Wolf & Berger (364) were calculated to yield a half life of 67 days for carnitine in rats, providing an explanation for the failure to observe uptake of labeled potential precursors. In these studies, labeled glycine or methionine, when administered for nine days, did introduce some radioactivity into isolated carnitine. Carnitine derived from glycine- 1-C^{14} was predominantly chain-labeled while that derived from methionine-methyl- C^{14} was predominantly methyl-labeled. Slow synthesis of carnitine in the intact rat was therefore demonstrated by these observations. Bremer (365) similarly obtained labeled carnitine from methyl-labeled methionine in the rat.

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CHEMISTRY AND FUNCTION OF POLYPEPTIDE HORMONES^{1,2,3}

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Advances in the peptide hormone field justify a review devoted exclusively to this rapidly expanding topic. The complete amino acid sequences of several peptide hormones are known and some of these structures have been verified by synthesis. The production, in the laboratory, of polypeptides with adrenocorticotrophic activity, two of them essentially as potent as natural ACTH, is a significant recent development. Impressive progress in peptide synthesis and purification has opened almost unlimited possibilities for structural variations. Although of very recent origin, this approach has already been used to identify certain structural features which are essential for biological function. Since the synthetic approach allows a degree of detailed dissection of peptide sequences not attainable by any other available technique, it can be expected to provide additional significant information in the future.

Workers in the physiological, biological, and chemical fields, who must contend with problems of endocrine homeostasis, have been greatly handicapped by the lack of chemically characterized tropic hormones.

In reviewing recent developments from a critical standpoint special emphasis will be placed on structure-function relations as derived from the biological evaluation of synthetic derivatives or fragments of certain polypeptide hormones. Some facets discussed in this review have been covered previously, but for the sake of clarity it seems desirable to bring some of them into focus with more recent advances.

¹ This review deals with oxytocin, the vasopressins, vasotocin, the melanocyte-expanding hormones, the corticotropins, the corticotropin-releasing factors, and parathyroid hormone. Most of the literature cited appeared prior to July 1961, but selected references of more recent date and data, as yet unpublished, are included.

² The following abbreviations are used: ac (acetyl); but (α -aminobutyric acid); cbzo (carbobenzoxy); CRF (corticotropin-releasing factors); form (formyl); MSH (melanocyte-expanding hormone). D-amino acids are shown in bold face type (Table I).

³ The author wishes to express his gratitude to Dr. Howard Rasmussen for the data presented in Tables III and IV and for additional results, as yet unpublished, pertaining to parathyroid hormone. He is much indebted to Dorothy Thompson for searching the literature and for checking of the bibliography and to Judy Smelas for her aid in getting the manuscript into its final form. He expresses his thanks to Drs. T. S. Danowski and P. G. Katsoyannis for many helpful discussions and for the critical reading of the manuscript.

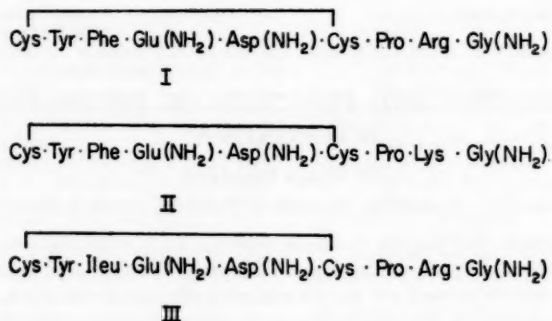


FIG. 1. Amino acid sequences of arginine vasopressin (I); lysine vasopressin (II); and arginine vasotocin (III).

SPECIES VARIATIONS

The vasopressins and vasotocin.—The presence of oxytocin in extracts of pig (1), beef (2), horse (3), sheep (4, 5), chicken (6), and human (7) pituitary glands is well established, and evidence is available to suggest that oxytocin is also present in pituitary extracts of certain fishes and frogs (8). A procedure based on an initial precipitation of the hormones in the form of a complex composed of oxytocin, vasopressin, and protein (van Dyke protein, neurophysin) has greatly facilitated the isolation of these hormones from small amounts of starting material (9). The peptide hormones are readily dissociated from this complex and can then be isolated by ion exchange chromatography. This procedure is also applicable to preparative purposes (10).

The chemical structures of three additional neurohypophyseal hormones (Fig. 1) illustrate the species variations which have been encountered. Amino acid interchanges are observed in positions 3 and 8. Arginine vasopressin has been isolated from beef (11), horse (3), sheep (5), and human (7) pituitary extracts, and has been identified by pharmacological techniques in pituitary extracts from many other species (12). Lysine vasopressin is a characteristic neurohypophyseal principle of the domestic pig (13).

Arginine vasotocin is a naturally occurring hypophyseal hormone, apparently characteristic of most nonmammalian vertebrates. Synthesis (14, 15) of vasotocin was achieved prior to isolation from *Rana esculenta* pituitaries (16).

It has been known for some time (17, 18) that extracts of fish, amphibian, avian, and reptilian pituitaries exert more powerful effects on water balance in the frog than can be attributed to their oxytocin and vasopressin content. Indeed, preparations from these species are more powerful in this respect than are similar materials from mammalian pituitaries. The activity of the latter coordinates well with the oxytocin and vasopressin content. Similar

observations are recorded with respect to sodium transport in isolated frog skin (natriferic activity) (19); here again, extracts from frog pituitaries are much more potent than can be predicted from the oxytocin and vasopressin content. At first, these findings led to the postulate that pituitary extracts from these nonmammalian species contain a substance differing from oxytocin and vasopressin. This hypothetical hormone was named "water-balance principle" or "natriferin" by different authors (17, 19). However, it has recently become apparent that the pharmacological spectrum of synthetic arginine vasotocin (20 to 23) parallels very closely that of nonmammalian pituitary extracts. Hence, the conclusion is almost inescapable that water-balance principle, natriferin, and arginine vasotocin are one and the same compound (24).

Some evidence has been presented for the presence of both arginine vasopressin and arginine vasotocin in chicken pituitary extracts (25). Since arginine vasotocin accounts for only one-half of the natriferic potency of *R. esculenta* pituitary extracts, the presence of another, as yet uncharacterized, natriferic principle is postulated (26). A detailed review of this subject has appeared (12).

Melanocyte-expanding principles.—Extracts from pig, beef, horse, monkey, and human pituitaries bring about darkening of frog skin both *in vivo* and *in vitro*. Fractionation of such extracts led to the isolation of three types of polypeptides, α -MSH, β -MSH, and corticotropin, all having the ability to expand melanocytes. The amino-acid sequences of these substances are illustrated in Figures 2 and 3. The hormones contain the heptapeptide sequence—Met.Glu.His.Phe.Arg.Try.Gly—which appears to be intimately connected with their ability to expand melanocytes. The amino-acid sequence of α -MSH is identical with that of the 13 N-terminal amino-acid residues of the corticotropins, but its N-terminus is acetylated and the chain terminates with a valine carboxamide group. Species variants of α -MSH are unknown. The same polypeptide is present in pituitary extracts from pig (27, 28, 29), beef (30, 31), horse (32), and monkey (33) glands. Melanocyte-expanding principles of the β -type have been isolated from pig (34 to 40), beef (41, 42), horse (43, 44), monkey (33), and human (45 to 48) pituitaries. The amino acid sequence of these hormones exhibits species differentiation (Fig. 2). The human hormone, a straight-chain polypeptide of 22 amino acid residues, is the largest structure. It differs from the monkey hormone by addition to the N-terminus of the tetrapeptide sequence, Ala.Glu.Lys.Lys. The monkey and human hormones differ from those of the other species by a lysine-arginine substitution. The differences between the pig and beef sequences involve a glutamic acid-serine exchange. The equine hormone contains an arginine moiety instead of a proline in the third to the last amino acid from the C-terminus. The melanocyte-expanding hormones of the β -type are less active biologically than α -MSH (XXVII, Table I). The following *in vitro* activities in MSH units/g are recorded: pig, 5×10^3 (30); beef, 2×10^3 (30); monkey, comparable to that of bovine β -MSH (33); horse, $\sim 2 \times 10^3$ (44).

Corticotropins.—The biological activities and amino acid sequences of

TABLE I
BIOLOGICAL ACTIVITIES OF PEPTIDES RELATED TO α -MSH AND TO THE CORTICOTROPINS

No.	Peptide	MSH U/gm	ACTH U/mg	Refs.
I	H ser. tyr. ser. met. glu. his. phe. arg. try. gly. lys. pro. val. gly. lys. arg. arg. pro. val. lys. val. tyr. pro. phe 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 39	$\sim 10^8$	80-100	
II	H ser. tyr. ser. met. glu. his. phe. arg. OH NH ₂	—	—	(135)
III	H phe. arg. try. gly. lys. pro. val. (NH ₂) form	—	—	(136)
IV	H his. phe. arg. try. gly. OH	2.3×10^4	—	(74, 137, 138)
V	H his. phe. arg. try. gly. OH	$\sim 10^8$	—	(136)
VI	H his. phe. arg. try. gly. OH	3.3×10^8	—	(139)
VII	H his. phe. orn. try. gly. OH	2.9×10^4	—	(140)
VIII	H his. phe. orn. try. gly. OH NH ₂	2.6×10^4	—	(140)
IX	H glu. his. phe. arg. try. gly. OH	2×10^8	—	(141)
X	H gly. his. phe. arg. try. gly. OH	2×10^8	—	(142)
XI	H met. glu. his. phe. arg. try. gly. OH NH ₂	1.4×10^8	—	(143)
XII	H met. glu. his. phe. arg. try. gly. OH NH ₂	2.8×10^8	—	(144, 145)
XIII	Chzo. ser. met. glu. his. phe. arg. try. gly. OH NH ₂	7×10^8	—	(135, 146)
XIV	H ser. met. glu. his. phe. arg. try. gly. OH NH ₂	7×10^8	—	(135, 146)
XV	Ac. ser. tyr. ser. met. glu. his. phe. arg. try. gly. OH NH ₂	3.6×10^8	—	(90, 147)
XVI	H ser. tyr. ser. met. glu. his. phe. arg. try. gly. OH NH ₂	—	—	(144)
XVII	H ser. tyr. ser. met. glu. his. phe. arg. try. gly. OH tos	2.9×10^8	—	(90, 147)
XVIII	H his. phe. arg. try. gly. lys. pro. val. (NH ₂) NO ₂ form	5×10^8	—	(74, 90, 135)
XIX	H his. phe. arg. try. gly. lys. pro. val. (NH ₂)	5×10^8	—	(90, 148)

TABLE I—(Continued)

No.	MSH U/gm	ACTH U/mg	Refs.
XX	8×10 ⁴		(90, 148)
XXI	8×10 ⁴		(90, 147)
XXII	5×10 ³		(149)
XXIII	~10 ³		(149)
XXIV	1.5×10 ³		(74, 75, 90)
XXV	2.0×10 ³		(75, 90, 150)
XXVI	2.2×10 ¹⁰		(75, 90)
XXVII	2×10 ¹⁰		(27, 76, 151)
XXVIII	1.9×10 ³	<0.1	(90, 147, 149)
XXIX	1.5×10 ³	—	(80)
XXX	2.0×10 ³	—	(80, 152)
XXXI	3.7×10 ³	<0.1	(80, 90, 152)
XXXII	~10 ⁷	29	(153, 154)
XXXIII		20-30	(155)
XXXIV		111.0±18.0	(80, 156)
XXXV	2.0×10 ³	—	(78)
XXXVI		<1	(80)
XXXVII	2.0×10 ³	103±10.3	(78)
XXXVIII			(79, 157)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

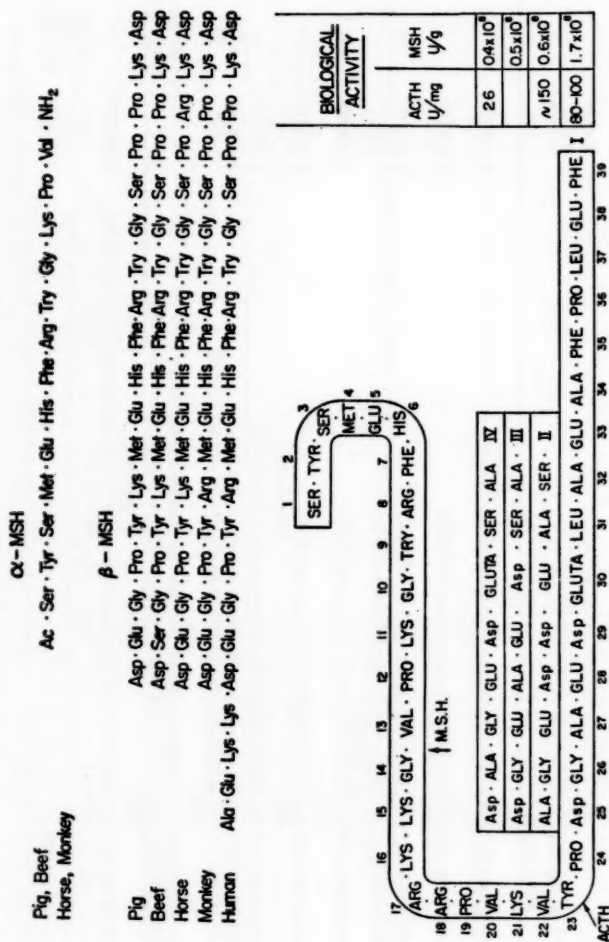


Fig. 2. (upper) Amino acid sequences of melanocyte-expanding hormones.

Fig. 3. (lower) Amino acid sequences of pig (I), sheep (II), beef (III), and human (IV) corticotropin.

pig (49, 50, 51), sheep (52 to 55), beef (56, 57), and human (47, 58, 59) corticotropins are shown in Figure 3. Species variations occur in those portions of the molecule (positions 25 to 33) which appear to be nonessential for biological activity. The "active" fragment (positions 1 to 24) is identical in all of the corticotropins which have been investigated to date. Discrepancies concerning the arrangement of amino acids between positions 25 to 30 in the pig hormone (49, 50, 51) have been reinvestigated, and the correctness of the formulation shown in Figure 3 appears to be firmly established (60). The relatively minor variations of the human hormone from those of the pig and sheep fail to provide a satisfactory explanation for its low biological activity (58). The reason may lie in the instability of the pure hormone in the highly dilute solutions employed for assay, or in partial oxidation of the methionine sulfur during isolation.

Two corticotropins, with identical amino acid composition but differing chromatographically and electrophoretically, can be isolated from porcine oxycel ACTH by ion-exchange chromatography (61). These peptides, corticotropin A₁ and A₂, differ solely by their amide content. The structure of corticotropin A₁ is shown in Figure 3; corticotropin A₂ contains a free glutamic acid carboxyl group in position 30. This modification explains the higher anodic migration rate of A₂ on starch gel electrophoresis (pH 8.5). The adrenal ascorbic acid-depleting activity of A₁ and A₂ (30 to 40 units/mg) is rather low in comparison to that of corticotropin A (100 to 150 units/mg) (62, 63). The latter material appears to contain mainly A₁ and a small proportion of A₂ (64), whereas β -corticotropin (65) is rich in A₂ but also contains some A₁ (64). The chemical nature of the various by-products (α , γ , and δ fractions) obtained in the course of isolation of β -corticotropin by countercurrent distribution (65) remains to be established. These materials, reported to possess adrenal ascorbic acid-depleting activity of 80 to 100 units/mg, exhibit the following steroidogenic potencies in units/mg: α_1 , 11.2 ± 2.1 ; α_3 , 34.0 ± 2.8 ; α_4 , 25.8 ± 5.1 ; γ_1 , 11.6 ± 2.0 ; γ_2 , 21.7 ± 4.1 ; δ , 27.6 ± 6.9 (66). β -Corticotropin possesses a steroidogenic potency of 94.5 ± 10.6 units/mg (66). The adrenal ascorbic acid-depleting activity is 80 to 100 units/mg (65). Highly purified ovine ACTH assays at 100 units/mg (67); this value appears more reasonable than the earlier "optimistic" figure (52). Reinvestigation of pig oxycel preparations by carboxymethyl-cellulose chromatography has revealed the presence of minor adrenocorticotropically active fractions, designated as A_{1a} and A_{1b}, in addition to A₁ and A₂ (68). The chemical nature of these compounds is unknown, but it appears likely that they differ from A₁ and A₂ in the functionally nonessential C-terminal sequence. They may correspond to some of the fractions obtained by countercurrent distribution (65).

SYNTHETIC PITUITARY HORMONES

Since the early syntheses of oxytocin (69) and the vasopressins (70, 71), impressive improvements in the methodology of peptide synthesis have

taken place; the more recent syntheses of these hormones (72, 73) have proceeded with excellent yields and afforded materials possessing a high degree of homogeneity. The basic principle which underlies all of the synthetic schemes involves preparation of protected straight-chain nonapeptide amides embodying the desired sequence. The protecting groups are then removed by reduction with sodium in liquid ammonia, and ring closure is brought about by air oxidation. Thus, ring closure depends on formation of a disulfide linkage and not of a peptide bond. The various analogues listed in Table II were prepared according to this principle.

Biological evaluation of the first synthetic peptide (74, 75) which embodied the entire amino acid sequence of α -MSH provided the initial clues pertaining to the nonessential nature for melanophoretic potency of the free γ -carboxyl group of glutamic acid and the free ϵ -amino group of lysine.

The observation that a synthetic peptide with the amino-acid sequence proposed for α -MSH (I, Fig. 4 and XXVII, Table I) possessed essentially the same biological activity and other properties of the natural material substantiates the chemical constitution of this hormone (76). Equally as active biologically as α -MSH is a synthetic derivative of this hormone (II, Fig. 4 and XXVI, Table I) in which the glutamic acid residue is replaced by glutamine and the lysine ϵ -amino group is substituted by a formyl group (75). This compound brings about marked darkening of the skin when administered to man. The biological activities of a variety of synthetic peptides related to the amino acid sequence of α -MSH are listed on Table I.

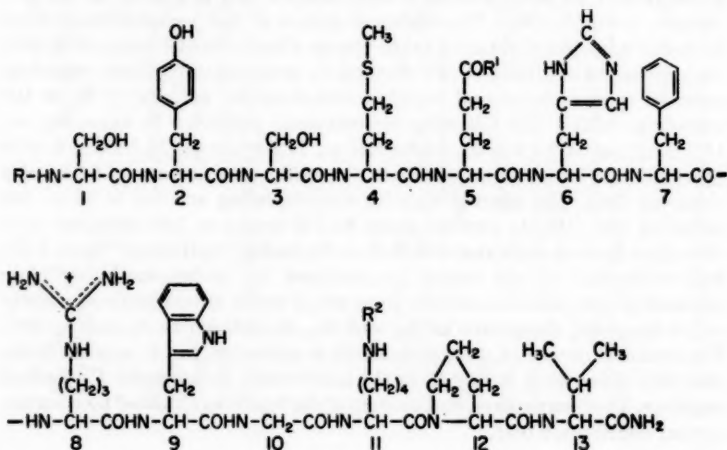


FIG. 4. Structure of α -MSH (I: R=acetyl; R'=OH; R $_2$ =H) and of a biologically fully active synthetic derivative (II: R=acetyl; R'=NH $_2$; R $_2$ =formyl).

Synthesis of a hormone of the β -MSH type has not yet been accomplished, but a few derivatives related in sequence to beef β -MSH and with weak melanocyte-expanding activity are known (77).

The tricosapeptide (I, Fig. 5 and XXXVII, Table I) constitutes the first synthetic peptide which possesses essentially the full adrenocorticotrophic activity of the corticotropins (78). The high degree of biological potency of this peptide establishes the concept, previously stated by inference (79), that the unit which is essential for full adrenocorticotrophic activity is located within the N-terminal tetracosapeptide fragment of the corticotropin molecule. The observations that the synthetic peptide produces a single spot on paper chromatography, that it travels as a single component on paper electrophoresis at various pH values, that it behaves as a homogeneous material on starch gel electrophoresis at pH 8.5 (80), and that, on acid hydrolysis, it liberates the constituent amino acids in the ratios expected by theory provide convincing evidence for its homogeneity. The fact that the entire molecule is assembled from two subunits of established stereochemical homogeneity by a step which is not likely to cause racemization (activation of a carboxyl group derived from glycine) and the finding that serine, glutamic acid, histidine, phenylalanine, arginine, lysine, proline, and valine in acid hydrolysates are of the L-variety (microbiological assay) support the stereochemical homogeneity of this synthetic ACTH fragment (80). The adrenal ascorbic-acid depleting potency of the synthetic peptide is 103 ± 10.5 units/mg (P 95 limits 84.8 to 126 units/mg) (80). Its steroidogenic activity (elevation of plasma corticosterone in the hypophysectomized rat) in two assays was estimated as 76 and 90 units/mg, respectively (P 95 limits 66 to 83 and 64 to 126 units/mg) (H. S. Lipscomb, personal communication). The synthetic ACTH peptide exhibits *in vitro* adrenocorticotrophic activity and is highly effective in releasing fatty acids from rat epididymal fat tissue *in vitro* (81). The ability of the peptide to bring about stimulation of the human adrenal cortex is discussed elsewhere in this review. In addition to its high level of adrenocorticotrophic potency, the synthetic compound has melanophoretic activity which is of the same order of magnitude as that of ACTH (see Table I). The protected tricosapeptide amide (II, Fig. 5 and XXXV, Table I), an intermediate in the synthesis of the active tricosapeptide, shows little if any adrenocorticotrophic activity but exhibits the same melanocyte-expanding potency as the free tricosapeptide. The synthetic tricosapeptide shares with other pepsin- or acid-degraded corticotropin fragments (corticotropin B type) the property of exhibiting a lower biological potency when administration is by the subcutaneous or intramuscular routes. The subcutaneous potency of the peptide approximates 24 to 28 units/mg (80). Thus, the potency ratio for this compound (subcutaneous/intravenous) is approximately 0.3/1; by definition the U. S. standard exhibits a ratio of 1/1 (see a later section). The reverse prevails in the case of corticotropin A.

TABLE II
BIOLOGICAL ACTIVITIES OF OXYTOCIN AND VASOPRESSIN ANALOGUES

No.	Oxytocin	Oxytocic Activity (U/mg)									Vasopressin Activity (U/mg)			Refs.		
		Uterus									Blood Pressure		Anti Diuresis			
		Cat <i>in situ</i>	Rat <i>in vitro</i>	Avian Depressor	Milk Ejection	Rat	Cat	Rat								
I	Cys-Tyr-Ileu-Glu(NH ₂)-Asp(NH ₂)-Cys-Pro-Leu-Gly(NH ₂)	1	2	3	4	5	6	7	8	9	—	—	—	(102)		
II	Cys-Tyr-Ileu-Glu(NH ₂)-Asp(NH ₂)-Cys	—	—	—	—	—	—	—	—	—	~ 3.3	0	~ 1.1	—	(102)	
III	Arg-vasopressin	—	—	—	—	—	—	—	—	—	~ 20.0	~ 60	~ 70	350-410	~ 400	(71, 103)
IV	Lys-vasopressin	—	—	—	—	—	—	—	—	—	5 ± 0.5	40 ± 5	—	268 ± 19	306 ± 13	(70, 104, 105)
V	Leu.	—	—	—	—	—	—	—	—	—	20	45	—	3	5	(108)
VI	His.	—	—	—	—	—	—	—	—	—	20	30	—	3	—	(105)
VII	Arg-vasotocin	—	—	—	—	—	—	—	—	—	~ 1.5	~ 4.6	—	1.5	—	(107)
VIII	Lys-vasotocin	—	—	—	—	—	—	—	—	—	~ 75	~ 150	~ 100	~ 125	—	(14, 15)
I	Oxytocin	—	—	—	—	—	—	—	—	—	20 ± 2	54 ± 4	55 ± 5	30 ± 4	38 ± 4	(105)
IX	Ileu.	—	—	—	—	—	—	—	—	—	—	100	—	130	—	(106)
X	Val.	—	—	—	—	—	—	—	—	—	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	(105)
XI	Leu.	—	—	—	—	—	—	—	—	—	563 ± 74	498 ± 37	328 ± 21	6 ± 1	—	(109)
XII	Leu.	—	—	—	—	—	—	—	—	—	380 ± 40	280 ± 17	310 ± 20	9 ± 1	—	(109)
V	Oxypressin	—	—	—	—	—	—	—	—	—	200 ± 15	280 ± 17	207 ± 14	~ 0.2	—	(109, 110, 111, 112)
XIII	Pha.	—	—	—	—	—	—	—	—	—	50 ± 8	57 ± 4	207 ± 14	~ 0.2	—	(109, 110, 111, 112)
XIV	Tyr.	—	—	—	—	—	—	—	—	—	45 ± 7	42 ± 1	101 ± 13	5	—	(109, 110, 111, 112)
	Tyr.	—	—	—	—	—	—	—	—	—	20	45	—	3	—	(106, 113)
		—	—	—	—	—	—	—	—	—	0.1 ± 0.03	0.03	1.5 ± 0.3	~ 0.01	~ 0.01	(105, 111, 112)
		—	—	—	—	—	—	—	—	—	0.04	0.1	0.1	0	0	(114)
		—	—	—	—	—	—	—	—	—						(115)

TABLE II—(Continued)

No.		Oxytocic Activity (U/mg.)		Vasopressin Activity (U/mg.)		Anti Diuresis	Refs.	
		Uterus		Milk Ejection	Blood Pressure			
		Cat <i>in situ</i>	Rat <i>in vitro</i>		Rat			Cat
XXXIX	His.Ser. --- Lys. ---	—	<0.01	—	0.01	<0.01	—	(115)
XXX	--NMe-Tyr.Ileu. --- Leu. ---	—	1.2±0.4	0.32±0.05	—	—	<0.001	(116)
XXXI	---Tyr.Phe. --- Lys.Ser(NH ₂) ---	—	0	0.01	—	0.4-0.5	—	(120)
XXXII	--OMe-Tyr.Ileu. --- Leu. ---	—	~5	~5	—	Inhibitory	—	(121)
XXXIII	---Tyr.Ileu. --- Leu. ---	—	—	Inactive	—	0	Inactive	(122)
XXXIV	---Tyr.Ileu. --- Leu. ---	—	—	Inactive	—	0	Inactive	(123)
XXXV	---Ileu(NH ₂) --- Leu. ---	—	—	Inactive	—	—	Inhibitory	(124, 125)
XXXVI	Gly.Cys.Tyr. --- Desamino oxytocin	—	Some	Inhibitory	—	Some	Inactive	(126)
XXXVII	β -MPRO* - Ileu. --- Leu. ---	—	698	578	—	1.2	—	(127)
XXXVIII	MAC† --- Ileu. --- Desamino lysine vasopressin	—	—	Inactive	Inactive	—	Inactive	(127)
XXXIX	β -MPRO* - Phe. --- Lys. ---	—	—	—	—	125	—	(127)
XL	Cys.Tyr.Tyr.Ileu.Glu(NH ₂).Asp(NH ₂).Cys.Pro.Lys.Gly(NH ₂)	—	—	Inhibitory	—	—	—	(128)
XLI	Cys.Tyr.Tyr.Phe.Glu(NH ₂).Asp(NH ₂).Cys.Pro.Lys.Gly(NH ₂)	—	—	Low Activity	—	—	Low Activity	(129)
XLII	Cys.Tyr.Tyr.Tyr.Glu(NH ₂).Asp(NH ₂).Cys.Pro.Lys.Gly(NH ₂)	—	—	—	—	—	—	(130)
XLIII	Cys.Tyr.Tyr.Phe.Glu(NH ₂).Asp(NH ₂).Cys.Pro.Lys.Gly(NH ₂)	—	—	—	—	Inactive	—	(131)
XLIV	Cys.Tyr.Tyr.Ileu.Glu(NH ₂).Asp(NH ₂).Cys.Pro.Lys.Gly(NH ₂)	—	—	—	—	—	—	(132)

* β -MPRO for β -mercaptopyroline acid.

† MAC for mercaptosuccinic acid.

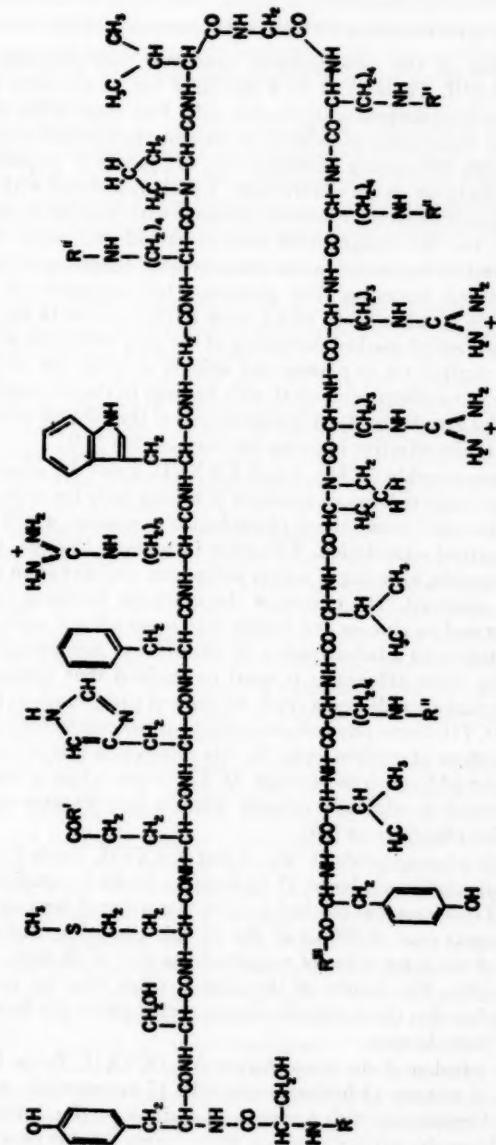


Fig. 5. Structure of segment of ACTH possessing essentially full adrenocorticotrophic activity (I: R = H; R' = OH; R'' = H; R''' = OH) and of an intermediate in its synthesis (II: R = NH₂; R' = NH₂; R'' = formyl; R''' = NH₂).

* β -MPRO for β -mercapto propionic acid.
† MAC for mercaptoacetic acid.

EFFECTS IN MAN OF SYNTHETIC PEPTIDES RELATED TO α -MSH AND ACTH

Administration of the homogeneous synthetic tridecapeptide amide (I, Fig. 4 and XXVI, Table I) (4 to 8 mg daily for 14 days) to humans brings about a striking darkening of the skin (82). This observation points to the physiological importance of α -MSH in human pigment-cell regulation. Although markedly influencing pigmentation, the synthetic peptide exerts no discernible effects on serum electrolytes. A patient afflicted with vitiligo (83) responded to administered synthetic hormone with marked darkening of the normal skin, but the depigmented areas remained unchanged. Urinary 11-oxysteroids and 17-ketosteroids are not increased following administration of the synthetic hormone. The protected tricosapeptide (II, Fig. 5 and XXXV, Table I), when given to a human at the level of 14 mg per day for one week, also caused marked darkening of the skin with little alteration of either serum electrolytes or plasma and urinary steroids (84, 85). These observations are in excellent agreement with findings in the rat, where these protected peptides are also ineffective stimulators of the adrenal cortex (80). Porcine β -MSH is also effective in darkening human skin (82).

The free tricosapeptide (I, Fig. 5 and XXXVII, Table I), when administered intramuscularly to humans at a level of 6.4 mg daily for seven days in a gelatin base, brought about those physiological responses which characterize adrenal-cortical stimulation. Elevation in urinary 11-oxy-, 17-keto-, and 11-desoxy steroids, lowering of serum potassium, and elevation of serum total CO_2 were observed. The effects of the synthetic hormone on serum sodium retention and on glucose and insulin tolerances are not equivalent to those observed following administration of 200 units of commercial ACTH (83). In assessing these differences it must be realized that comparison is made between a pure peptide and a crude mixture of compounds as it occurs in commercial ACTH. Some physiological effects of the commercial preparation may not be those of corticotropin. In this connection it is of interest to note that partial acid hydrolysis of crude ACTH causes a loss of the antidiuretic activity which is originally present, but the hydrolysates appear to retain their clinical effectiveness (86).

The synthetic tricosapeptide (I, Fig. 5 and XXXVII, Table I) brought about marked stimulation of plasma 17-hydroxycorticoids in a patient whose endogenous ACTH release was blocked by administration of dexamethasone. A single intravenous dose of 300 μg of the peptide elicited a response that was essentially of the same order of magnitude as that of 24 units of commercial ACTH (81). The results of the clinical trials thus far conducted allow the conclusion that the synthetic tricosapeptide exerts marked adrenocorticotrophic activity in man.

Intravenous infusion of the nonadecapeptide (XXXII, Table I) brings about elevation of urinary 17-hydroxycorticoids, 17-ketosteroids, and aldosterone in man. Comparison with a sample of purified porcine corticotropin suggests a steroidogenic activity of about 24 units/mg for this peptide (87).

A single intramuscular dose of 150 μ g of the nonadecapeptide failed to elicit a response. This is not surprising, since this peptide is likely to share with other adrenocorticotropically active fragments of the corticotropin molecule (corticotropin B type) the characteristically low activity ratio (subcutaneous/intravenous).

The observation that exposure to dilute hydrochloric acid converts the adrenocorticotropically inert, melanophoretically active, peptide (XXXV) into a peptide (XXXVII) which possesses both potent adrenocorticotropic and melanocyte-stimulating activity provides an interesting example of how chemical manipulation may affect physiological activities (80).

OXIDATION-REDUCTION BEHAVIOR OF CERTAIN PEPTIDE HORMONES

Exposure to such oxidizing agents as hydrogen peroxide or performic acid abolishes the adrenal-stimulating activity of the corticotropins, (88, 89) and the melanocyte-expanding potency of the α -MSH derivative (II, Fig. 4 and XV, Table I) is markedly reduced, but not eliminated, by this treatment (90). Porcine β -MSH is also subject to reversible inactivation by oxidation (91), and a similar behavior is observed with the parathyroid peptides (92, 93, 94). Incubation with such thiol reagents as cysteine or thioglycolic acid brings about complete reactivation of the oxidized form of all of these hormones. The oxidized form of ACTH can be separated from the reduced species by ion-exchange chromatography (61, 89, 95). The finding that Raney-nickel-exposed corticotropin retains the ability to undergo reversible oxidation-reduction led to the erroneous conclusion that the methionine sulfur was not involved (96). The Raney-nickel-treated hormone was assumed to contain α -aminobutyric acid rather than methionine. It has since been observed (97) that the Raney-nickel treatment had failed to bring about desulfurization of the hormone (the reasons for this are not clear) and that the methionine sulfur is indeed responsible for the reversible inactivation-reativation of the ACTH molecule.

Studies with synthetic peptides support this conclusion (80). Hydrogen peroxide converts the pentapeptide, Ser.Tyr.Ser.Met.Glu(NH₂), into an oxidation product which differs markedly in R_F from the starting material. Methionine sulfoxide, and not methionine, is present in leucine-aminopeptidase digests of the oxidized peptide, while the other amino acids remain unchanged. Thus, the expected behavior of a methionine peptide is observed, i.e., ready conversion to the corresponding sulfoxide analogue. On the other hand, the octapeptide amide, His.Phe.Arg.Try.Gly.(ϵ -formyl)Lys.Pro.Val(NH₂), undergoes no significant change upon exposure to hydrogen peroxide. The behavior on paper chromatography, the ultraviolet spectrum, and the amino acid composition of leucine-aminopeptidase digests of the original and the oxidized peptide are indistinguishable. These experiments show clearly that serine, tyrosine, glutamine, histidine, phenylalanine, arginine, tryptophan, glycine, proline, and valine in the peptidic combinations which exist in the MSH and ACTH molecules are not subject to oxidation by hydrogen

peroxide. On the other hand, methionine in the very association in which it is present in these hormones forms the sulfoxide. Since the model octapeptide contains N⁶-formyllysine, and not lysine, no definitive statement can be made pertaining to the stability of the N⁶-amino group to peroxide oxidation. It seems highly unlikely, however, that this function would undergo reversible oxidation-reduction. The conclusion seems inescapable that the methionine sulfur is the locus, in ACTH and the melanocyte-expanding hormones, which is responsible for their characteristic oxidation-reduction behavior.

The ACTH-inactivating system of dog plasma is heat labile and thus appears to be enzymatic in nature. The rate of inactivation is temperature-dependent and L-cysteine inhibits the process. The possibility exists that the *in vivo* inactivation involves an oxidative process, but more evidence will be necessary to establish this point (98, 99).

The close correlation between residual biological activity and methionine sulfoxide content of samples of a parathyroid peptide (partially oxidized with

TABLE III
SUMMARY OF PREPARATION AND PROPERTIES OF PARATHYROID POLYPEPTIDES

Extraction Procedure	Phenol			Acetic Acid			HCl	
Peptide	A	B	C	C'	D	E	F	G
Isolation Method	Sepha- dex	Sepha- dex	ccd*	ccd*	ccd*	ccd*	ccd*	ccd*
Purity†								
ccd	×	—	×	×	—	—	—	—
Sephadex	×	×	×	×	—	×	×	—
Chromatography								
a) paper	×	×	×	×	×	×	×	×
b) partition (column)	×	—	×	—	—	×	×	×
Dialysis	×	—	×	×	—	—	—	—
Paper Electrophoresis	—	—	—	—	×	—	—	×
Ultracentrifugation	—	—	×	—	×	—	×	—
End Group								
N-terminal	Alanine	—	Alanine	Alanine	—	—	Valine	—
Molecular Weight	8500	7200	8500	8500	6900	5600	5200	3800
Biological Activity								
Calcium Mobilizing	2750	2250	2750	2550	1500	1050	900	850
Phosphaturic	—	—	3000	—	1400	800	—	—

* CCD for countercurrent distribution.

† X indicates that purity was evaluated by the test indicated.

TABLE IV
AMINO-ACID COMPOSITION OF PARATHYROID POLYPEPTIDES

Peptide	A	B	C	C'	D	E	F	G
Amino Acid	Residues per molecule of polypeptide							
Lysine	7	7	7	7	6	4	4	3
Histidine	3	4	3	3	2	3	2	1
Ammonia	—	—	7	7	6	4	3	2
Arginine	4	3	4	4	3	2	2	2
Aspartic Acid	8	6	8	8	6	5	4	3
Threonine	1	2	1	1	2	2	2	1
Serine	7	4	7	7	5	4	3	3
Glutamic Acid	10	7	10	10	8	5	5	4
Proline	3	2	2	2	2	2	2	1
Glycine	4	4	4	4	4	4	3	2
Alanine	6	8	6	6	6	6	6	3
Valine	6	6	6	6	4	3	3	2
Methionine	2	1	2	2	2	1	1	1
Isoleucine	3	2	3	3	2	1	1	1
Leucine	7	7	7	7	6	5	5	3
Tyrosine	1	1	1	1	1	1	1	1
Phenylalanine	2	2	2	2	2	2	2	1
Tryptophan	1	1	1	1	1	1	1	1
Total	75	67	74	74	62	51	47	33

H₂O₂) containing only one methionine residue (See Tables III and IV) locates the oxidation-reduction center of this hormone in the methionine sulfur atom (94). Only one of the two methionine moieties in the larger parathyroid peptides seems to be intimately connected with biological activity (see section on parathyroid hormone). It is of particular significance that the methionine sulfur appears to play a vital role in the function of those peptide hormones that exhibit such a wide spectrum of biological action as do the corticotropins, the melanocyte expanders, and the parathyroid hormones. Structure-function relations (to be discussed in a later section) have demonstrated that methionine is not necessary for melanocyte-stimulating activity. Its ability to enhance this particular activity is attributable to more favorable binding of the methionine-containing peptides to the receptor site (64). The exact relation between function and the methionine sulfur, as concerns adrenocorticotrophic and parathyroid activity, remains to be clarified.

STRUCTURE-FUNCTION STUDIES

General comments.—Accurate knowledge of structure appears to be basic to an understanding of the mechanism of action of biologically active substances. This necessitates precise knowledge of the chemical nature of the

functional groups and, in the last analysis, of the spatial relation of every atom with respect to all other atoms. To date, x-ray diffraction of crystals provides the most accurate information on structure in the solid state. The structure of complex molecules in aqueous solution is largely unknown.

The group of middle-sized polypeptide hormones which is under consideration has thus far resisted crystallization, and the x-ray diffraction method cannot be applied to the study of their structure. Two crystalline salts of oxytocin, the flavianate (1) and the *p*-hydroxyazobenzene-*p'*-sulfonate (100) may be amenable to x-ray diffraction studies. The repeating peptide unit provides the backbone of the peptide hormones, and the side chains characteristic of the constituent amino acids constitute the functional groups.

Hopefully, evaluation of the relations between amino acid sequence and biological activity may lead to recognition of those portions of the hormone molecules which provide the key to function. Unfortunately, the influence of structural alterations on physiological activity cannot always be described in precise quantitative terms, and hence assessment of their fundamental importance becomes at best an arbitrary matter. This is particularly true in instances where substitutions of specific amino-acid residues or other structural modifications bring about a decrease, but not a complete elimination, of activity. An analogue which exhibits low activity in respect to its therapeutic value may still appear to retain the structural prerequisites for "function" although higher concentrations may be required to achieve a biological effect. It is this very aspect of the problem which is extremely difficult to assess.

At present, biological evaluation of peptide-hormone fragments, or analogues, involves assays with intact animals or *in vitro* systems such as isolated organs or tissue slices still possessing a high degree of organization. Many factors which may influence the apparent biological activity must be considered. These include the rate of transport from the site of administration to the receptor molecule or molecules, the rate of transport through cellular membranes, the rate of inactivation, the time required for onset of a maximal response, and possibly other factors. Since variants of the hormones may be affected differently by these phenomena, it becomes difficult to assess their absolute potency with respect to a standard. In the past, very similar compounds were compared during standardization; in such situations one might expect identical behavior of standard and unknown (dose response slopes, etc.). These same assumptions may not be valid when a pure analogue or fragment of a peptide hormone is compared with a crude standard preparation. Observations in the corticotropin series are illustrative. The USP standard of this hormone possesses, by definition, a 1/1 ratio of subcutaneous to intravenous potency. Compared to this standard, subcutaneously administered corticotropin A is approximately three times as potent as intravenously administered hormone. Conversely, fragments of the corticotropin molecule of the corticotropin B type which possess full adrenal ascorbic acid-depleting activity when given intravenously may possess as little as 25 per cent of full activity when administration is by the subcutaneous or intra-

muscular routes. It would appear that differences in the rates of absorption, transport across membranes, time of onset of maximal response, or inactivation are responsible for this behavior.

Awareness of these points is implicit in the following discussion which summarizes current information concerning structure-function relationships in the oxytocin-vasopressin, melanocyte-expanding and adrenocorticotrophic hormone series.

The oxytocin-vasopressin series.—The biological spectra of a series of analogues of oxytocin and vasopressin are presented in Table II. The present discussion will focus only on the most fundamental points which relate structure and function. Two recent reviews (12, 101) provide information on pharmacological details. Most of the biologically active analogues exhibit both oxytocic and vasopressor activity, a property which is characteristic for the parent hormones (133, 134). The studies which provide the basis for the following discussion are listed in Table II.

The 20-membered ring system of oxytocin minus the sidechain (II) possesses a very low level of oxytocic, and no measureable vasopressor, potency. From these results it would appear that the sidechain is essential for both types of activity.

Both the glutamine residue in position 4 and the asparagine moiety in position 5 are critical for biological activity. This follows from the observation that substitution of glutamine by isoglutamine (XXXV), of asparagine by isoasparagine (XXXIV), and of asparagine by glutamine (XXXIII) brings about complete inactivation. Elongation of the peptide chain at the amino end by glycine (XXXVI) represents another modification which is incompatible with physiological activity. Substitution of the peptide bond hydrogen derived from the tyrosine in position 2 (XXX) or that derived from the glycine in position 9 (sarcosine analogue) (XXXI) results in marked depression of oxytocic activity in the former and total loss of biological function in the latter. A variant of the oxytocin molecule which commands particular attention is desaminooxytocin (XXXVII). Here the amino group derived from the cysteine residue in position 1 is replaced by β -mercapto-propionic acid. This substance exhibits higher oxytocic activity than does oxytocin itself, both as concerns rat uterus and avian depressor activity. This result decisively eliminates the free amino group from those functionalities which are essential for biological activity. The loss of biological function which accompanies acylation of the amino group by glycine (XXXVI) may involve interference with the attachment of the oxytocin molecule to its receptor site. Replacement of the cysteine in position 1 by mercaptoacetic acid (XXXVIII) destroys biological activity, an observation which provides convincing evidence for the critical importance of the 20-membered ring. Analogues in which an additional amino acid residue is introduced into the ring between positions 2 and 3 (XL to XLV) are inactive.

Various amino acid substitutions involving positions 2, 3, and 8 have been performed. Biological activity seems dependent on the presence in position 2

of either a tyrosine or phenylalanine residue. Replacement of either of these moieties by serine (XXIII and XXV) or histidine (XXVII) brings about total inactivation. Replacement of tyrosine by phenylalanine [compare (I and XVIII) or (IV and XXI)] lowers biological activity but is compatible with function. It thus appears that the phenolic hydroxyl is not essential for, but enhances, biological activity. In this connection it is worthy of note that the 2-*p*-methoxyphenylalanine analogue of oxytocin (XXXII) is considerably less active than the 2-phenylalanine analogue (O-methyl-oxytocin) (XVIII). This may be explicable in terms of steric interference of the bulky methoxyl group with the hormone-receptor interaction, but the possibility has not been ruled out experimentally that the phenylalanine analogues may undergo hydroxylation when in contact with tissues and that their biological activity is the result of this biological transformation. Parahydroxylation is impossible in O-methyl-oxytocin.

Comparison of the oxytocic activity of the parent hormone (I) with analogues (XI to XIV) illustrates the effects of variations in position 3. Biological activity in this series decreases in the order ileu > val > leu > phe > tyr > try. The last-mentioned analogue is devoid of activity, the second last possesses a very low order of oxytocic potency. The same trend is also apparent in the 2-phenylalanine oxytocin series (XVIII to XX). Here again, biological activity decreases in the order ileu > phe > tyr, with the tyrosine analogue being essentially inert. It is worthy of note that whereas parahydroxylation of a phenylalanine residue in position 2 enhances markedly biological activity, the opposite effect occurs when a phenylalanine moiety occupying position 3 is replaced by tyrosine. Analogues (XIII, XV, XX, and XXII) in which tyrosine occupies the 3 position are almost devoid of biological activity.

It has been noted that replacement of the isoleucine residue in position 3 by valine or leucine lowers oxytocic activity. On the other hand, substitution of the leucine residue in position 8 by isoleucine (IX) exerts little effect on potency; on the contrary, the activity of this substance appears to be at least equivalent, or even superior, to that of the parent hormone. Substitution of the 8-leucine by valine (X) brings about a general decrease in biological potency.

Turning to the relationships between structure and vasopressin activity, one notes that alterations which bring about elimination of oxytocic activity also destroy vasopressor potency. Apparently, there exists a close parallelism between certain essential structural features which are important for both physiological activities. The single outstanding structural alteration which brings about conversion of predominantly oxytocic compounds into substances possessing primarily vasopressor activity appears to involve substitution of the leucine residue in position 8 by arginine or lysine. Invariably, the arginine analogues are more effective vasopressor agents than are the corresponding lysine derivatives. Arginine vasopressin (III) is more active than lysine vasopressin (IV), and arginine vasotocin (VII) is more potent than ly-

sine vasotocin (VIII). This relation appears to hold also as far as rat anti-diuresis is concerned.

Histidine vasopressin (VI), an analogue of vasopressin in which the leucine in position 8 is replaced by histidine, possesses only weak vasopressor activity. It would appear that vasopressor activity varies in accordance with the base strength of the R-group derived from the amino acid which occupies position 8, but the fact that the leucine derivative (V) is more active than the histidine analogue (VI) is hardly explicable in these terms.

The second, but less important, substitution which favors vasopressor over oxytocic activity involves the exchange of the isoleucine in position 3 by phenylalanine. This is apparent when one compares the vasopressor activity of the two vasopressins (III and IV) which contain the phenylalanyl moiety with that of the vasotocins (VII and VIII). These latter compounds contain an isoleucine residue in position 3. Again, as noted previously in connection with the discussion of the oxytocin analogues, vasopressor activity is markedly diminished when the tyrosine in position 2 is replaced by phenylalanine (compare IV and XXI). Analogues in which the aromatic amino acids in position 2 are replaced by serine or lysine are inactive. Parahydroxylation of the phenylalanine moiety in position 3, i.e., the replacement of phe by tyr, brings about essentially total inactivation of the molecule. It has been noted that replacement of the N-terminal amino group in oxytocin by a hydrogen atom has little effect on biological activity (desaminoxytocin). Similarly the N-terminal amino group is not essential for vasopressor potency, as is apparent from the high blood pressure activity of compound (XXXIX) in the rat. However, this analogue appears to be less active than its amino analogue lysine vasopressin.

Antagonists for oxytocin or vasopressin are of practical importance, but attempts to discover potent compounds possessing this biological characteristic have thus far met with unimpressive results. A number of analogues (XXXII, XXXV, XXXVI, XL, and XLIV) appear to possess limited antagonistic properties. Thus, the isoglutamine analogue of oxytocin (XXXV) inhibits the pressor activity of administered vasopressin in the anesthetized cat (125) and glycyloxytocin (XXXVI) blocks the action of oxytocin on avian blood pressure (126). The compound fails to inhibit the effect of oxytocin on the isolated rat uterus.

O-Methyl-oxytocin (XXXII) is a peptide which is endowed with anti-vasopressor activity despite the fact that it possesses limited oxytocic potency (121). A homologue of oxytocin in which an additional tyrosine moiety is interposed between the tyrosine in position 2 and the isoleucine in position 3 (XLIV) is also antagonistic. This peptide blocks the action of oxytocin as concerns avian blood pressure and rat uterus contraction, but the compound stimulates milk ejection in the lactating rabbit (132).

The α -MSH corticotropin series.—The studies which are summarized in Table I provide the basis of the following discussion, certain aspects of which have been summarized previously (80, 90).

The observation that an octapeptide fragment (XIII, XIV) from the center of the α -MSH sequence possesses the ability to expand melanocytes *in vitro* prompted the testing of a series of polypeptides structurally related to α -MSH and to the corticotropins. The results of these investigations led to the conclusion that certain, but not all, fragments of the α -MSH molecule are active biologically. The smallest peptide fragment thus far described which possesses melanocyte-expanding activity is the pentapeptide (IV) but its activity is only approximately one-millionth that of the parent hormone. The fact that the biological activity of this peptide has been observed in different laboratories points to its fundamental significance and suggests that peptide (IV) embodies within its structure the very unit which is concerned with function.

It is apparent from inspection of the data in Table I that melanocyte-expanding activity reaches its maximum at the tridecapeptide stage and that it decreases gradually as the peptide chain is elongated from the carboxyl end. Melanophoretic activity reaches the level of the corticotropins (approximately 10^8 units/g) with a chain of 23 amino-acid residues (XXXV and XXXVII). Not all fragments of the α -MSH molecule are endowed with melanocyte-expanding properties. Hence, chain length alone cannot explain this biological function. For example, the octapeptide (II) which corresponds to the N-terminus of the α -MSH molecule is inactive, but addition of tryptophylglycine gives a biologically active peptide (XVI). The C-terminal heptapeptide amide (III) is inert but biological activity ensues when a single histidine residue is attached to its N-terminus (XX). These findings suggest key functions for histidine and tryptophan as concerns melanocyte-expanding potency. Whether or not the glycine in position 10 is concerned with melanophoretic activity remains to be established.

The observation that melanocyte-expanding hormones of both the α - and β -type and the corticotropins embody within their structures the heptapeptide fragment Met.Glu.His.Phe.Arg.Try.Gly (XI) prompted speculations that this fragment provides the key to their biological properties. The observation that peptides (XX) and (XXI), which do not contain the entire heptapeptide sequence, exhibit higher biological activity than does peptide (XI) suggests that the unit which is essential for melanophoretic activity resides in the pentapeptide sequence of peptide (IV).

The arginine moiety within the above-mentioned sequence may not play an important role, since its replacement by ornithine (VII) in the pentapeptide (IV) exerts little, if any, effect on biological activity. Similarly, replacement of arginine by nitroarginine (XX and XIX), although decreasing biological activity, appears compatible with function.

The heptapeptide (XI) is apparently more active than its glutamine analogue (XII), but it is evident that with highly active peptides such as (XXVI and XXVII) glutamine is equivalent to glutamic acid as far as melanophoretic activity is concerned. This is not too surprising, since substitution of glutamine by glycine (IX and X) exerts no influence on biological activity.

Obviously, this conclusion is based on the experimentally unfounded assumption that structure-function relations which are observed with the small, low-potency peptides are applicable to the highly active, more complex substances. Exposure of the α -MSH derivative (XXVI) to hydrogen peroxide brings about diminution, but not complete elimination, of biological activity from 10^{10} to approximately 10^7 MSH units/g. Incubation with thioglycolic acid or cysteine restores biological potency (90). In conjunction with the observation that a number of peptides not containing methionine possess the ability to expand melanocytes, this finding eliminates the methionine sulfur as an indispensable site for biological function. The impressive activity of the α -aminobutyric analogue (XXIX) supports this conclusion. The high level of melanocyte-expanding activity of the various hormones of the β -MSH type (see section on species variations) which differ markedly in amino acid sequence from α -MSH indicates that chain length is more important than a highly select sequential arrangement of amino acids in the nonessential portion of the molecule, i.e., the pentapeptide sequence covering positions 6 to 10. It should be remembered, however, that all the hormones possess the same central heptapeptide sequence.

Derivatives of α -MSH in which the N-terminus is acetylated appear to be more active than their counterparts that possess a free N-terminal amino group. In the peptide pairs (XXII, XXIII), (XXVII, XXVIII), and (XXX, XXXI), the acetylated compound is the more active one. However, some of the peptides are not strictly comparable, since they differ with respect to other protecting groups, i.e., glutamine amide and N'-formyl group on lysine. It should be noted, however, that peptides (XXXV) and (XXXVII) are equally active, although the former contains an acetylated N-terminus and the latter a free N-terminal serine. The presence of an N-terminal carbobenzoxy group is compatible with melanophoretic activity, but the activity of the carbobenzoxy derivative is lower than that of the corresponding acetylated peptide (XXIV) and (XXV). The melanocyte-expanding activity of the octapeptide (XIV) is not altered by carbobenzoxylation of the amino group (XIII).

Biological evaluation of derivatives of peptide hormones in which various functional groups are substituted may provide information pertaining to the essential nature of these groups for biological activity, but the results of such studies must be interpreted cautiously, since the observed biological activity may reflect the ability of the test object to remove the protector groups. Evidence, to be presented later, shows that neither the rat nor the human appears to be capable of removing N'-formyl groups from the ACTH sequence. It seems likely that frog skin also lacks this ability. Thus, the high level of biological activity of a number of peptides, notably (XXV and XXVI), in which the N'-amino group of the lysine is protected by a tosyl or a formyl moiety, respectively, eliminates this amino group as an element essential for function. The size of the tosyl group seems to be responsible for the lower biological activity of (XXV) with respect to (XXVI).

It appears likely that exposure to alkali, which brings about a prolongation of the darkening effect of α -MSH and ACTH in the hypophysectomized frog (30, 51, 158), may involve racemization of certain amino acid residues in the peptide chain. The observation that exposure to alkali prolongs the melanophoretic activity of (IV) (30, 138) indicates that this phenomenon may be explicable in terms of structural alterations in this particular section of the MSH and ACTH molecules. It is of interest to note that three analogues (V, VI, and VIII) of this pentapeptide in which phenylalanine and arginine are replaced by the respective unnatural amino acids possess essentially the same activity as the 4-L form; in the case of peptide (VI), the activity is even higher. Two of these (IV and VII) show potentiation following exposure to alkali. Peptide (VIII) is inactivated and the biological activity of peptide (VI) is unchanged by this treatment. The chemical mechanism which is responsible for the prolongation effect remains to be discovered.

Evaluation in the rat, by the *in vivo* ascorbic acid-depletion assay, of a series of synthetic peptides related structurally to the corticotropin molecule has delineated unequivocally that section of the molecule which is responsible for adrenocorticotrophic activity. It remains for future studies to discover the essential nature of individual amino acid residues within this functional unit.

The heptapeptides (XI and XII), the decapeptide (XVII), and the acetylated tridecapeptide (XXVI) are inactive. The tridecapeptide amide (XXVIII) is the shortest fragment of the ACTH sequence which possesses a low but significant and reproducible adrenocorticotrophic activity. Not only does this substance bring about adrenal ascorbic acid-depletion, but it stimulates steroidogenesis as well. It is the first synthetic peptide reported in the literature which possesses both adrenocorticotrophic and melanophoretic activity. Elongation of its chain from the carboxyl end by the Gly.Lys.Lys moiety (XXXI) fails to increase adrenocorticotrophic activity. The adrenal ascorbic acid-depleting potency of the hexadecapeptide amide (XXXI) is essentially the same as that of the tridecapeptide. The protected tridecapeptide amide (XXIX) and the protected hexadecapeptide amide (XXX) are inert as far as ascorbic acid depletion or steroidogenesis is concerned, but both compounds are highly effective expanders of melanocytes.

Adrenocorticotrophic activity increases sharply when the chain reaches the nonadecapeptide stage. The glutamic acid and glutamine peptides (XXXII and XXXIII) have essentially the same ascorbic acid-depleting activity. Both compounds are considerably less potent than peptide (XXXVII). The melanophoretic activity of the glutamic acid peptide is considerably lower than would be expected from comparison with other peptides possessing an even longer amino-acid chain. The eicosapeptide (XXXIV) is reported to possess an *in vitro* adrenocorticotrophic activity of 2 to 3 units/mg (156). Data regarding *in vivo* potency of this compound are unavailable. The finding that peptides possessing a shorter amino acid chain exhibit 25 to 30 per cent of the rat adrenal-ascorbic-depleting activity of natural ACTH suggests the same or a higher potency for the eicosapeptide. Indeed, crude preparations

of the amide of this compound prepared by a route which differs markedly from that used previously (159) possessed an *in vivo* potency at the level of 31 ± 5 units/mg. The pure eicosapeptide possessed a potency of 111.0 ± 18.0 units/mg.

Thus, essentially, full adrenocorticotrophic potency is reached when the peptide chain is extended to 20 amino acid residues [peptide (XXXIV)]. The synthetic approach to the study of the relation of structure and function in the corticotropin series has shown that the unit of the corticotropin molecule which is endowed with full adrenocorticotrophic activity is longer than 16 amino acid residues and may be shorter than 20 amino acid residues. The finding that activity increases sharply when the Arg.Arg.Pro moiety is added to the C-terminus of the 16 amino acid chain establishes the key importance for high-level activity of the Lys.Lys.Arg.Arg fragment which is located in positions 15 to 18. The protected tricosapeptide amide (XXXV) exhibits a very low level of ascorbic acid-depleting activity (0.05 to 0.1 units/mg), but the stimulation resembles a non-dose-related all-or-none response type. A dose of 0.05 mg of the protected peptide, administered by the subcutaneous route, failed to produce a significant alteration in the ascorbic acid level, but a significant rise in plasma corticosterone (10.3 to $15.0 \mu\text{g}$ per 100 ml of plasma) ensued and continued to be maintained for three hours (80). The resting level of corticosterone in 24-hour hypophysectomized rats is 4 to 6 μg per 100 ml of plasma.

The protected tricosapeptide is highly active as far as melanocyte-expanding activity is concerned. It is worthy of note that the various blocking groups, i.e., the N-terminal acetyl, the glutamine amide, and the formyl group, are among a class of substances which are normally present in cells, and pathways are available for their attachment to and elimination from other molecules. It is thus surprising to find that neither the rat nor the human possesses the ability to convert the biologically inert protected tricosapeptide amide into the adrenocorticotropically active deblocked tricosapeptide (XXXVII).

The rate of inactivation of corticotropin during leucine aminopeptidase digestion appears to parallel the rate of the release of the N-terminal serine (160). Selective acetylation of the N-terminus (161) brings about a striking lowering in ascorbic acid-depleting activity and an increase in melanophoretic activity. Also, exposure of ACTH to the action of periodate (162) brings about inactivation. All this evidence points to the importance of the N-terminal serine amino group for adrenocorticotrophic activity. The finding that the partially protected tricosapeptide amide (XXXVI) possesses only a very low biological potency compared to the totally unblocked peptide (XXXVII) focuses attention on some or all of the free lysine amino groups as essential for adrenocortical stimulatory potency. The biological testing of derivatives of the tricosapeptide-containing blockers attached to the ϵ -amino group of select lysine residues may identify the essential amino groups.

Present experimental evidence, presumably supporting the view that a

free γ -carboxyl group in position 5 of the corticotropin molecule is required for high-level adrenocorticotropic activity, is unconvincing (163). Exposure of beef corticotropin to the action of succinic anhydride at pH 9.0 brings about a considerable reduction of both adrenocorticotropic and melanocyte-expanding activities (164). The availability of melanocyte-expanding hormones and corticotropically active synthetic peptides of defined purity and structure has facilitated exploration of the biological spectra of these hormones. The present study is limited to discussion of the relation between amino acid sequence and melanocyte-expanding and adrenocorticotropic activity. However, it has become increasingly apparent that in addition to their effects on pigment cells, the melanocyte-expanding hormones elicit other physiological responses. β -MSH appears to possess central-nervous-system stimulatory activity in the cat (165, 166) and both α -MSH and β -MSH are reported to accelerate the rate of disappearance of radioactivity from the rabbit thyroid following a tracer dose of I^{131} (167). Intracisternal injection of corticotropin or α -MSH into cats and dogs elicits in these animals "a crisis of increasing muscular tonus spreading to many different muscular groups and resulting in a generalized act of stretching." This effect appears to be specific for the two mentioned hormones; a number of other pituitary factors are inactive (168). The synthetic α -MSH derivative (XXVI, Table I) brings about the same phenomenon but appears to be somewhat less effective (169). Acceleration of the heart rate in a dog heart-lung preparation is observed following administration of natural α -MSH, corticotropin, or of the synthetic peptide (XXVI, Table I) (170).

An impressive amount of evidence [summarized in a recent review (171)] demonstrates that ACTH exerts a number of extra adrenal actions.

CORTICOTROPIN-RELEASING FACTORS (CRF's)

Corticotropin releasers are defined as substances (presumably of hypothalamic origin) which appear to be the ultimate mediators for activation of the adenohypophysis to release corticotropin when an animal is exposed to stress (172). Compounds designated as corticotropin-releasing factors must act directly on the adenohypophysis and bring about the release, or increased biosynthesis, of corticotropin (173). Animals with median eminence lesions or animals in which the release of corticotropin is blocked by administration of steroids or morphine-nembutal are used for the *in vivo* testing of these factors. Such animals, possessing an intact pituitary, fail to release ACTH when stressed, but respond to administered corticotropin-releasing substances. The *in vitro* test system of Saffran & Schally (174, 175, 176) provided the key to the detection and isolation of CRF. The natural vasopressins, because of their ability to bring about corticotropin release *in vitro* and *in vivo*, were implicated as the normal physiological agents which regulate ACTH release (177 to 182).

However, rather convincing evidence is now available to show that sub-

stances present in extracts of the hypothalamic area or the neurohypophysis and which possess low vasopressor activity are highly powerful in releasing ACTH (183). Concentrates containing CRF can be differentiated from vasopressin, oxytocin, ACTH, histamine, acetylcholine, epinephrine, and 5-hydroxytryptamine by their pharmacological and chromatographic behavior (184, 185, 186). Such concentrates exhibit CRF activity both *in vitro* (186, 187) and *in vivo* (186, 188). Hog posterior pituitary powder has served as the starting material for most of the fractionation studies, but CRF-active concentrates have also been prepared from median eminence extracts derived from beef, rat, and calf brains (189, 190).

Two distinctly different types of CRF-active material appear to be present in posterior lobe powder (191, 192). One of these, a basic peptide, exhibits a behavior akin to that of α -MSH and for this reason is designated as α -CRF. A fraction highly enriched in this material exhibited CRF activity *in vitro* and *in vivo* at a dose of 0.5 and 2.0 μ g, respectively. This material possessed some adrenocorticotrophic (0.1 units/mg), vasopressor (0.1 to 0.2 units/mg), and a rather high level of MSH activity (1 to 3×10^9 units/g). The peptide nature follows from the observation that α -CRF liberates amino acids on hydrolysis with acid. In addition to those amino acids which constitute the α -MSH molecule (29), threonine, leucine, and alanine are present in the hydrolysate. The sequential arrangement of the amino acids is unknown.

An apparently homogeneous CRF-active peptide which may be identical with α -CRF, contains threonine and leucine in addition to the amino acids found in α -MSH (193, 194). This peptide exhibits only 1 to 2.5 per cent the melanocyte-expanding potency of α -MSH.

It is of interest to note that pure α -MSH (XXVII, Table I) and the synthetic derivative (XXVI, Table I) elicit no CRF response *in vitro*, but fragments of their amino acid sequence (XI, XII, and XVI) are reported to be highly effective *in vitro* (143, 145, 194). However, the *in vitro* CRF activity of the small peptides could not be confirmed (195), and the heptapeptide (XII) shows neither adrenal nor pituitary stimulation in man at a dose level of 6 mg (196). The synthetic tricosapeptide (XXXVII, Table I) elicits marked adrenal stimulation in man in a single intravenous dose of 300 μ g (81).

The exact structural prerequisites for α -CRF activity remain to be elucidated, particularly with reference to those features which differentiate CRF and melanocyte-expanding activity.

The second type of CRF, designated as β -CRF (184, 188, 191, 192), differs markedly from α -CRF and appears to possess a significantly higher biological potency (50 to 100 times that of the α -type). Highly purified samples are effective at dose levels of 0.05 μ g *in vitro* and 0.1 μ g *in vivo*. β -CRF contains cystine, and its properties are very similar to those of vasopressin with which it associates intimately during fractionation. It is regarded as a small peptide, possibly an analogue of vasopressin (192).

PARATHYROID HORMONE

Comprehensive reviews of this subject have appeared (94, 197). Available evidence (198, 199) supports the view that both calcium-mobilizing and phosphaturic activities of bovine parathyroid extracts reside in a single peptide molecule. The exact sequential arrangement of the amino acids in this peptide remains to be established, but highly homogeneous preparations are at hand.

A family of structurally related peptides (Table III) ranging in molecular weight from 3800 to 8500 can be isolated from parathyroid extracts by procedures involving salt and solvent extractions, ultrafiltration, precipitation with trichloroacetic acid, and countercurrent distribution (94). The amino acid composition of these compounds is shown on Table IV. The most active parathyroid-hormone preparations possess approximately 2800 units of calcium-mobilizing and 3000 units of phosphaturic activity (198, 199).

The procedure which is employed to liberate the active hormone from glandular tissue to which it is tightly bound significantly influences biological activity and molecular size of the peptides which can be obtained. The largest and most active peptide, apparently the genuine parathyroid hormone (peptides A, C, and C', Table III), can be isolated from phenol extracts of bovine glands (199, 200, 201). Preparations that are apparently identical may be obtained by countercurrent distribution (peptide C and C') or gel-filtration techniques (94) (peptide A). An active material of lower potency and molecular weight (peptide B) has also been isolated from certain phenol extracts (94). The amino acid composition (Table IV) of this material differs somewhat from that of the apparently genuine hormone peptide. Fractionation of the parathyroid-active materials in 80 per cent acetic-acid extracts of bovine glands (202) led to isolation of a biologically active peptide (peptide D, Table III) which appears to represent a slightly degraded form of parathyroid hormone. Peptide C (Table III) has also been obtained from such extracts (94). Three peptides of still lower molecular weight but possessed of biological activity are found in 0.2 *N* hydrochloric acid extracts of bovine glands (203, 204, 205). Pure parathyroid preparations have not been isolated from the tissues of other species. Extensive evaluation of the purity of the various peptides indicates a high degree of homogeneity.

The fact that parathyroid hormone does not contain cystine and the presence of a single alanine N-terminus in the molecule point to a straight-chain polypeptide structure (199). Alterations of the N-terminus appear to be compatible with physiological activity. This follows from the observation that valine and not alanine provides the N-terminal end of one of the smaller but biologically active molecules. The losses which are observed during purification of parathyroid-active extracts are largely the result of an oxidative process involving conversion of the two methionine residues to the sulfoxide (92, 93, 94). Biological activity is regained when inactive preparations are incubated with thiol reagents. From the amino acid analyses

(Table IV) it is apparent that the smallest biologically active subunit of parathyroid hormone, presently recognized, contains only one of the two methionines which are present in the intact hormone. It is this particular methionine moiety which appears to be connected intimately with function (see section on oxidation-reduction).

Apparently, parathyroid hormone contains one or more rather labile peptide bonds which undergo cleavage with formation of the smaller active units under the conditions of the acetic- and hydrochloric-acid extractions. Apparently, these bonds are not sensitive to phenol. Thus, parathyroid hormone shares with α -MSH and the corticotropins the interesting feature that only a portion of the amino acid sequence appears necessary for biological function. The question as to why nature adds "bulk" to the functionally important segment of these molecules is indeed a challenging one.

FUNCTION

Understanding of function of a hormone in fundamental terms implies detailed understanding of the chemical events whose modulation provides the observed physiological response. Within this frame of reference the action of hormones is obscure. The results of structure-function studies which are reviewed point to specific relations between peptide sequence and physiological activity. Details of these relations are far from clear but certain trends are evolving. It appears that only a limited number of amino acid side-groups are intimately involved in the chemical event which is the key to physiological function and that large portions of the peptide molecule are concerned with binding of the functionally effective residues or "active sites" to the receptors.

We believe that function depends on a critically defined spatial orientation of the peptide hormone on the receptor molecule, and that specific sequential arrangements of amino acids are necessary for this to occur optimally. The combination of inactive S-peptide with inactive S-protein which leads to the creation of active ribonucleases (206) may provide a profitable working hypothesis pertaining to the function of the polypeptide hormones. We have stated (90),

It is highly tempting to speculate that the mode of action of the peptide hormones may involve their combination, in a highly selective manner, with a receptor protein to create an active enzyme. The section of the hormone peptide which we designate as the "active site" could thus become an integral portion of an active site in terms of enzymology.

We have defined the "active site" of a polypeptide hormone as that region of its sequence which is capable of functioning physiologically, although usually much less efficiently than the intact hormone. The available evidence points to His.Phe.Arg.Try.Gly (IV, Table I) as the active site for melanophoretic activity. The N-terminal tridecapeptide appears to represent the "active site" for adrenocorticotropic activity (XXVIII, Table I). We are

inclined to attribute the marked enhancement in adrenocorticotrophic activity which occurs upon addition of the Arg.Arg.Pro moiety to the hexadecapeptide (XXXI, Table I) to increased affinity for the receptor.

The property that significant portions of the α -MSH and corticotropin molecules are not essential for function is shared by parathyroid hormone. Here again, sizable portions of the molecule can be removed without total loss of physiological activity. The "active site" in this instance must be located within the sequence of the smallest active peptide.

Whereas covalent bond formation between the peptide hormone and receptor appears improbable as concerns the melanocyte-expanding, adrenocorticotrophic, and parathyroid hormones, there is experimental evidence available which suggests that the attachment of vasopressin to its receptors in rat kidney (207, 208) and the amphibian bladder (209, 210) may involve a disulfide linkage. It appears, however, that the strict structural requirements for vasopressor and antidiuretic activity, which have been delineated in another section, involve other structural features in addition to the disulfide bond. Indeed, the reversible binding of this hormone and of oxytocin to van Dyke protein (211) suggests that these compounds have the capacity to bind to proteins through noncovalent linkages.

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NUCLEIC ACIDS: MOLECULAR BIOLOGY OF DNA^{1,2}

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The molecular biology of DNA is a relatively new field and as such has been undergoing significant changes in a short time. Many results of just a few years ago may be considered out of date in that they are incomplete or incorrect. For this reason, the literature included in this article covers principally the last two years. We have attempted to be critical rather than exhaustive. No attempt will be made to define molecular biology in a sentence or two, since the field is broad and indeed it promises to envelop more and more biology as research efforts continue. While in some instances the topics discussed could be classified as purely physico-chemical, they are included because they form the foundation of what is to follow.

Because of the importance of the DNA molecule per se, it has become important to be able to define it explicitly and precisely, notwithstanding the fact that more than one type exists in any given DNA sample. This need has not always been appreciated. Up to the present, many investigations have been concerned with the bulk properties of a sample of DNA, but it is clear that if one wishes thereby to invoke a molecular interpretation of biological function, it is essential that the heterogeneity of the assembly of DNA molecules studied be understood in all physical and chemical respects. One major stumbling block has been aggregation. Among other things, we wish to be able to distinguish a natural aggregate, which of course has meaning, from an artificial one. For instance, is the single entity isolated from T2 phage (1, 2, 3) a single "real" molecule, a real aggregate, or an artificial aggregate? With regard to chemical heterogeneity, we would want to know, for example, if the base analysis of a synthetic DNA sample applies to each molecule present in the mixture. If it does, are all the base sequences identical? These are typical questions which must be dealt with if molecular biology is to be meaningful.

MOLECULAR WEIGHT AND AGGREGATION

A number of techniques for determining the molecular weight of DNA have been in use for many years. Among the oldest are viscosity and sedimentation velocity measurements, which require assumptions about shape and polydispersity before the molecular weight can be calculated. Recently, sedimentation constants have been most commonly employed to calculate molecular weights through empirical relationships, which have been derived for homologous series of degraded DNA's [Doty *et al.* (4); Cavalieri &

¹ The survey of literature pertaining to this review was completed November 1961.

² The following abbreviations will be used: DNase (deoxyribonuclease); RNase (ribonuclease).

Rosenberg (5)]. The application of these formulas to nonhomologous DNA samples, which may differ (for example) in polydispersity, is risky. In addition, it should be noted that there may be technical difficulties in determining the sedimentation constant, even with ultraviolet optics [Schumaker & Marano (6), Hearst & Vinograd (7)]. The methods of sedimentation equilibrium and approach to equilibrium also present technical problems. In the latter, the concentration gradient is difficult to measure accurately with absorption optics, and in the former, the wobble of the rotor at the necessary low speeds gives rise to convective disturbances. In the method of sedimentation equilibrium in a density gradient ("banding") (8), the problems of preferential solvation and density heterogeneity of the DNA prevent an explicit calculation of the molecular weight.

Light scattering is an absolute method which may be used to determine both the molecular weight and a characteristic dimension of the molecule. The method was first used for DNA by Doty & Bunce (9). The limitation of the method is set by the radius of gyration and not the molecular weight (10). Thus, the correct weight can be obtained for molecules of large radius of gyration provided measurements can be made at sufficiently small angles (11, 12). Ordinarily, angular measurements may be made to 20° and, for an incident beam of wavelength 5780 Å, extrapolation gives the correct weight-average molecular weight for molecules with a radius of gyration of about 2000 Å or less. Most DNA samples fall within this range. Sadron (12a) and Katz (16) made measurements down to 10° and 9° on DNA of 6 and 12 million molecular weight, and obtained the same weight as for measurements at angles greater than 30° .

A wide range of particle weights by light scattering has been reported, but the values have centered around 6 to 8 million, with some as high as 16 million [Shooter (13); Hermans (14); Fleischman (15)]. The problem of protein and metal ion contamination has been recognized [Butler, Robins & Shooter (17); Kirby (18)] and its role in molecular-weight determination has been emphasized recently by Cavalieri, Deutsch & Rosenberg (19). In a systematic study of molecular weights of DNA samples from seven sources, these authors have shown that the weights, after exhaustive disaggregation treatment, lie in the range 0.6 to 2 million. The possibility of degradation by enzymes or shear during isolation of these samples has been eliminated.

It has, however, been established that even low molecular weight DNA can be degraded by shear under certain conditions [Cavalieri (20); Cavalieri & Rosenberg (5)]. These results have been confirmed for DNA of high molecular weight, using other sources of mechanical shear (21, 22, 23). The physical studies of Fleischman (15) were apparently made on T2 and T4 DNA which had been inadvertently degraded by shear.

On the basis of chromatographic behavior on a basic protein column, Hershey & Burgi (22) have concluded that the initially homogeneous DNA particles from T2 phage are cleaved, upon stirring, into halves or quarters, depending on the speed of the stirrer. They used these results to calibrate

sedimentation and viscosity measurements in terms of relative molecular weight (24), obtaining an almost linear curve of $\log S$ versus $\log M$. It was necessary to make a more or less arbitrary assignment of one molecular weight. The choice was supported by the work of Thomas & Berns (25), who determined by banding in a density gradient that there were two DNA molecules per T2 phage, each of 62 million molecular weight. Later radioautographic estimations, however, showed that this was in error and that there is but one DNA particle of about 130 million molecular weight in T2 (1, 2). Beer came to the same conclusion on the basis of electron microscopic measurements (3). Light scattering has confirmed the weight of the undegraded particle (125 ± 25 million), which appears to have a compact configuration in solution; but the light scattering results have cast some doubt on the weight of the putative quarter molecules (26).

DENATURATION

As applied to DNA, denaturation is generally taken to mean a collapse of the rigid helical structure into a random coil. In some instances the process can be reversed to some extent, but this will be considered as a separate subject in the next section. Denaturation is a cooperative process which occurs when the equilibrium between intact and ruptured hydrogen bonds is shifted to the right beyond a certain critical point. Factors which can produce such a shift include ionic strength, pH, temperature, and dielectric constant of the solvent. The equilibrium situation exists only in native DNA, where broken sequences are, of course, short and where sufficient hydrogen bonds are always present to maintain the native helical structure [Cavalieri & Rosenberg (27); Zimm (28)]. The broken regions may be of significance in the replication of DNA.

It seems safe to conclude that all the various methods of denaturation result in the same denatured state, provided that the conditions are such as to avoid (or minimize) chemical degradation.

Denaturation of DNA has been studied by a number of physical techniques. These include: ultraviolet spectrophotometry [R. Thomas (29)]; infrared spectrophotometry [Kyogoku *et al.* (30)]; potentiometry [Cavalieri & Rosenberg (27); Cox & Peacocke (31)]; light scattering and viscosity [Reichmann, Bunce & Doty (32); Cavalieri, Rosoff & Rosenberg (33); Rice & Doty (34); Geiduschek (35)]; chromatography [Rosenkranz & Bendich (36)]; flow dichroism [Cavalieri, Rosenberg & Rosoff (37)]; optical rotation [Doty *et al.* (38)]; electron microscopy [Hall & Litt (39)]; sedimentation [Inman & Jordan (40, 41, 42, 43); Coates & Jordan (44)]; conductometric titration [Felsenfeld & Huang (45); Inman & Jordan (46)].

In an analysis of the ultraviolet spectrum of nucleic acids, Rich & Kasha (47) attributed the absorption at 260μ to $\Pi \rightarrow \Pi^*$ transitions in the planes of the bases and $n \rightarrow \Pi^*$ transitions, polarized perpendicular to the base planes. Alignment of $n \rightarrow \Pi^*$ moments should produce hypochromism. Tinoco (48) has developed equations for the degree of hypochromism resulting from these

dipole-dipole interactions in stacked parallel bases. His calculations account for the hypochromism actually observed in DNA (35 to 40 per cent). Since hypochromism is mainly a measure of the relative orientation of bases, the decrease in optical density is proportional to the number of bases ordered, but only if there are at least 7 or 8 residues in the ordered regions. Rich & Tinoco (49) discuss the effect of chain length on hypochromism, and an approximate equation for this effect is given by Applequist (50). It would seem from these considerations that one must be cautious in relating the degree of hyperchromism directly to the fraction of bases disordered or of hydrogen bonds broken during denaturation.

The temperature at which the denaturation of DNA occurs can be related to its base composition [Marmur & Doty (51)]. Apparently the guanine-cytosine pair is more stable to heat than the adenine-thymine pair. Marmur & Doty have obtained a linear relationship between the transition temperature (T_m) and the guanine+cytosine content of DNA over a range of 0 to 67 per cent guanine+cytosine. Unlike the T_m , the hyperchromic shift decreases with increasing guanine+cytosine content. The temperature range of the transition is an indication of the compositional heterogeneity of the DNA. In this connection it is to be noted that guanine+cytosine content is also related to density (52, 53), which fact may be used to determine the heterogeneity of DNA from various sources by means of the banding technique (52, 53). Sueoka (54) has recently compiled base compositions and densities for DNA from a number of sources.

Organic solvents such as formamide and dimethylsulfoxide denature DNA, as shown by the loss of secondary structure in these solvents [Helmkamp & Ts'o (55)]. When the DNA is returned to an aqueous solvent it remains denatured. On the other hand, while DNA in methanol or ethanol possesses a collapsed structure, as shown by light scattering, sedimentation, and viscosity measurements, when it is returned to an aqueous system the DNA appears to be undenatured [Herskovitz, Singer & Geiduschek (56)].

The principal biological assay for denaturation has been bacterial transformation [Zamenhof, Alexander & Leidy (57); Hotchkiss (58)]. Roger & Hotchkiss (59) showed that there are two different selective heat inactivation processes for pneumococcal transforming DNA. They define critical inactivation as involving the collapse of the entire molecule, corresponding to a helix-coil transition. Different drug-resistance markers had different critical inactivation temperatures. Below the melting point, sub-critical inactivation of the DNA was presumably due to depurination in small regions within the molecule. The order of inactivation of the markers by this process was different.

In a similar study of heat inactivation, Ginoza & Zimm (60) describe a collapse inactivation (helix-coil transition) and a single hit inactivation (depurination at temperatures below the melting point). Differential effects on various markers were noted. They deduced that the single-hit process takes place with both native and denatured DNA, and they calculated a

target size of 430 base pairs. The latter, however, must be accepted with caution in view of the ambiguities inherent in the assay system when there is any change in the physical properties of the DNA (61, 62).

Guild (63), preceded by Roger and Hotchkiss (59), has presented evidence that denatured DNA may possess transforming activity, the low levels observed being attributable to low absorption by the recipient cell. Ginoza & Guild (64) studied the effects of different methods of isolation of transforming DNA on its heat inactivation.

Inactivating agents other than heat have also been shown to exert a characteristically differential effect on different markers in transforming DNA [Litman & Ephrussi-Taylor (65); Lerman & Tolmach (66); Zamenhof (67)].

Recently, considerable emphasis has been placed on the hypothesis that the polynucleotide chains of DNA separate completely as a consequence of denaturation [Marmur & Lane (68); Doty, Marmur, Eigner & Schildkraut (10)]. It is currently quite common to find the terms "denatured" and "single stranded" used interchangeably. However, although the evidence of Marmur and coworkers is consistent with strand separation, it cannot be considered as proof. They observed a partial restoration of transforming activity when heat-denatured pneumococcus DNA was slow-cooled. This effect was concentration-dependent, and was increased by addition of unmarked homologous but not heterologous DNA. Band density measurements, absorbance-temperature curves, and electron microscopic observations confirmed that renaturation occurred on slow cooling of pneumococcus and other bacterial DNA's but not of DNA from calf thymus or salmon sperm. They found that their DNA was degraded as well as denatured by the heat treatment, and that aggregation occurred during renaturation. Nevertheless, they calculated a halving of the molecular weight on denaturation by extrapolating sedimentation and viscosity measurements to zero heating time and applying the Mandelkern-Flory equation in an empirical manner.

In contrast, light scattering shows that the molecular weight is unchanged by denaturation [Rice & Doty (34); Cavalieri, Deutsch & Rosenberg (19)], even when measured above the transition [Sadron (12a)]. When the light scattering measurements are carried out immediately after denaturation, no aggregation is observed in the concentration range 0.04 to 0.15 mg/ml (19); consequently, the apparent constancy of molecular weight cannot very readily be attributed to aggregation. In addition, a study of the decrease in molecular weight as a function of the number of bonds hydrolyzed by DNase II indicated that there was no change in the number of strands per molecule upon denaturation [Cavalieri & Rosenberg (69)]. Heat-denatured calf thymus DNA, for example, was found to possess approximately two strands per molecule. The kinetics of renaturation [Cavalieri, Small & Sarkar (70)] also indicate that denatured DNA molecules consist of more than one strand. Renaturation of heat-denatured *Escherichia coli* DNA follows first-order kinetics; this means, of course, that only one kinetic

entity is involved in the reaction and therefore the strands could not have come apart originally. The mechanism of the renaturation reaction will be discussed in detail in the following section.

Some recent results on strand separation in T2 and T4 phage DNA are also of interest. Berns & Thomas (71) have shown that T4 can be denatured in two minutes. If the molecular weight is 120 million (1, 71) then it would take at least 12.4 minutes for each molecule to unwind, according to the equation of Longuet-Higgins & Zimm (72); [also see Kuhn (73, 74)]. It has been emphasized that this is a minimum time (72); therefore, it seems unlikely on theoretical grounds that the strands separate upon denaturation.

Fortunately, all the results just cited can be accounted for in a straightforward manner, if denaturation is accompanied by a partial separation of strands, only. The evidence for this will be discussed in the next section. Since the physical properties of DNA from the bacteriophage Φ X-174 resemble those of denatured DNA,³ an unambiguous interpretation of the number of strands in each Φ X-174 DNA molecule is not possible at present.

RENATURATION

The mechanism of renaturation can be examined most fruitfully from the general viewpoint of helix-coil transitions, setting aside for the moment the question of the number of strands in the denatured molecule. There have been a number of theoretical treatments of this subject [Rice & Wada (75); Hill (76); Zimm & Bragg (77)]; those of Zimm (28) and Gibbs & DiMarzio (78) deal specifically with DNA. Briefly, the DNA molecule is considered as a linear array of bonds holding two strands together. Breakage of these bonds by, for example, increasing the temperature is accompanied by changes in entropy and free energy which are not necessarily proportional to the number of bonds broken. The distribution of breaks over the molecule as a function of temperature is calculated, and it has been shown that a sigmoid curve is to be expected when plotting the fraction of intact hydrogen bonds versus temperature. This accounts for the experimentally observed curves of DNA absorbance versus temperature. The theory also predicts that the transitions both from helix to coil (denaturation) and the reverse (renaturation) can be brought about by temperature changes.

Finally, it has been shown theoretically that it is possible through a

³ The density, radius of gyration, absorbance-temperature curve, and sedimentation behavior of Φ X-174 DNA, as well as its hyperchromicity, reactivity toward formaldehyde, and priming ability in the Kornberg system (133, 159), are like those found for denatured DNA (see, for example, 42) and, in some cases, unlike those found for single-stranded RNA. The greater efficiency of Φ X-174 inactivation by P^{32} decay (160), compared to the T-phages (161), does not constitute evidence for single-strandedness, since all these inactivations are single-hit. The efficiency difference can be explained by the great difference in DNA content. T2, for example, performs the same basic function, replication, as Φ X-174, yet contains 70 times as much DNA; this suggests that T2 DNA contains redundant or nonessential information.

kinetic approach to decide whether the renaturing species is a single unit or a number of units [Flory (79); Saunders & Ross (80)]. In this connection, it is of interest to note that collagen, which is a three-stranded helix, has first order renaturation kinetics, although the strands have been thought to separate at denaturation (81). Hodge *et al.* (82) have recently presented evidence that the renaturation observed is due to a minority of residual three-stranded denatured molecules. Ross & Sturtevant (83) have found that the formation of a poly (adenylate and uridylylate) double helix follows bimolecular kinetics, as is to be expected.⁴ In a similar manner it has been shown that the kinetics of renaturation of denatured DNA from *E. coli* follows first-order kinetics when the reaction is carried out in 1 M salt at 60°, 70°, and 80°C. [Cavalieri, Small & Sarkar (70)]. It can be concluded, therefore, that renaturation is not a process in which two single polynucleotide chains unite to form a double helix; rather, the renaturing unit must be a single kinetic entity. The latter must possess at least two strands, between which the original hydrogen bonds can reform. The recovery, on slow cooling, of transforming activity is undoubtedly due to such a zippering up of the denatured molecule, whose strands had not completely come apart but were held together by entanglements and residual hydrogen bonds formed randomly between bases.

When DNA is renatured at higher temperatures, on the other hand, there is an increasing second-order contribution to the kinetics (70). On the basis of present theory [Flory (79); Cavalieri, Small & Sarkar (70)], the rate can best be accounted for by simultaneous uni- and bimolecular reactions. The extent of contribution from each mechanism to the over-all renaturation process will depend on the temperature, among other variables. Thus, the bimolecular reaction appears to occur between single-stranded regions, probably chain ends, of two denatured, multistranded molecules. Approximate complementarity of base sequences is an apparent requirement for the formation of helical regions by either mechanism, judging from the specificity observed (84). The concentration effect on the recovery of transforming activity is undoubtedly due to this bimolecular interaction, which increases the molecular weight and thus the absorption of the transforming DNA by recipient cells (62).

The kinetic results are compatible with the observations of Marmur and coworkers (10, 84), in which renaturation of mixtures of N¹⁴- and N¹⁵-labeled DNA's led to the appearance of N¹⁴-N¹⁵ complexes when the DNA samples were homologous. The kinetics are also consistent with the results of Herriott (85), who showed with bacterial transformation that genetic linkages are formed when DNA samples containing certain single markers are renatured together. In other words, it appears unnecessary to invoke the existence of single-stranded DNA to account for complex (hybrid) formation by annealing.

⁴ At lower ionic strengths, the reaction deviated from second order kinetics.

A recent finding by Geiduschek (86) promises to shed light on some aspects of the renaturation of DNA as well as on the problem of chemical mutagenesis. By treatment of salmon sperm DNA with either nitrous acid or nitrogen mustard he obtained a product which could be reversibly heat-denatured as shown by optical density, viscosity, and band density measurements. For example, the absorbance-temperature curve was typically sigmoid, but on fast cooling the DNA returned to its original state, whence the same curve could be repeated. He suggests that these mutagens may bring about cross-linking, which could hold the DNA chains in register so that return to the original structure is facilitated. The action of ultraviolet light also renders DNA somewhat resistant to denaturation, presumably, because of cross-linking [Marmur & Grossman (87)].

These results probably explain why denatured salmon sperm and calf thymus DNA cannot ordinarily be renatured (10). Evidently these DNA's have base sequences of such a kind that the strands are readily knocked out of register, perhaps by looping out of one of the chains and mismatching in short regions. This would be prevented by cross-linking, thereby permitting renaturation as observed (86). In this connection, it is significant that the d-AT copolymer, which has a regularly-alternating sequence of adenylate and thymidylate in each chain, is also reversibly denatured (88). Bacterial DNA's, in which the base sequences are more complex and more difficult to match, do not renature completely even under optimal conditions (68). The DNA of higher organisms may be even more complex, thus obviating the argument that its inability to renature arises from the smaller fraction of complementary strands available for participation in a bimolecular renaturation reaction (10).

The effect of diamines ($H_3N^+(CH_2)_nNH_3^+$; $n=2$ to 8) on the transition temperature of calf thymus and T2 DNA has been examined by Mahler, Mahotra & Sharp (89). They note that the greatest increase in stability occurs when $n=5$. This type of observation may prove useful in structure studies.

DNA-RNA COMPLEXES; RNA SYNTHESIS

At the present time there is an increasing body of indirect evidence that (a) a certain RNA fraction (messenger RNA) functions to transfer information (90, 91, 92, 93, 94, 95) and (b) this RNA receives its information from DNA by means of a direct physical interaction at the time of RNA synthesis. These are both reasonable hypotheses, but they must still be regarded as such. We will discuss first the physical experiments which relate to the existence of DNA-RNA complexes, and then the biological evidence, including DNA-dependent RNA synthesis *in vitro*.

Interactions among polynucleotides were first reported by Felsenfeld *et al.* (96, 97). The single-chain polymers used as reactants were of the ribose type, and both two- and three-stranded helices were observed crystallographically [Davies & Rich (98); Rich (99); Rich *et al.* (100)]. These results

led Rich (101) to attempt to form helical structures in which one chain was a polyribonucleotide while the other was of the deoxyribose type. He used polyadenylic acid (2000 residues) and polydeoxyribothymidylic acid (12 residues) and concluded on the basis of spectrophotometric and sedimentation data that a hybrid helix was formed. The rationale behind these experiments was to explore the possibility of a physical basis for transfer of information by template action, and the results indicate that the possibility does exist.

In preliminary experiments, Schildkraut *et al.* (102) suggest the formation of a hybrid between polydeoxyguanylic acid and polyribocytidylic acid. One of the reactants was a synthetic helical complex of polydeoxyguanylic acid and polydeoxycytidylic acid. The second reactant was polyribocytidylic acid. The mixture was heated and annealed. The postulated reaction is (polydeoxyguanylic acid + polydeoxycytidylic acid) + polyribocytidylic acid \rightarrow (polydeoxyguanylic acid + polyribocytidylic acid) + polydeoxycytidylic acid. On the basis of the resistance of the product to both RNase and DNase, and the intermediate density of the single band obtained upon density banding, the investigators concluded that the product was (polydeoxyguanylic acid + polyribocytidylic acid). However, the densities of the bands of polyribocytidylic acid (polydeoxyguanylic acid + polydeoxycytidylic acid) and the product are respectively 1.88, 1.80, and 1.86. It would appear that the product density should be closer to that of (polydeoxyguanylic acid + polydeoxycytidylic acid) if, unlike polyribocytidylic acid, the product is helical. A second problem, noted by the authors, is the unknown fate of the polydeoxycytidylic acid which is presumed to have been displaced quantitatively; the excess polyribocytidylic acid is also missing in the band pattern.

Hall & Spiegelman (103) have performed similar experiments using naturally occurring nucleic acids. They annealed denatured T2 DNA with RNA which had been isolated from T2-infected *E. coli*. The RNA synthesized after infection is known to have the same base composition as T2 DNA [Volkin & Astrachan (90)]. This T2-specific RNA was labeled with P^{32} , and the DNA with tritium. When the mixture was banded in a cesium chloride gradient, a P^{32} peak was found to coincide with the tritium peak. This did not occur when the RNA was mixed with native T2 DNA or when it was annealed with DNA from *Pseudomonas aeruginosa* or T5 (which has the same gross base composition as T2 DNA). The weight ratio of RNA to DNA in the complex was estimated at a maximum of 1 to 5; thus it is not a simple hybrid but some type of aggregate, possibly similar to that formed in the bimolecular renaturation of DNA. Its formation appears to require a certain degree of homology.

What appear to be natural DNA-RNA complexes have been examined by Spiegelman, Hall & Storck (104). *E. coli* was infected with P^{32} -labeled T2 phage and pulsed with tritiated uridine to label the RNA. The cells were lysed and banded before the commencement of DNA synthesis. A tritium

peak, superimposed on a high density shoulder in the P^{32} band, was located at approximately the same density as the artificial complex discussed above (103). Material from the complex band was isolated and shown to contain both RNA and DNA by treatment with alkali, which labilized tritium but not P^{32} .

In another type of experiment Hayashi & Spiegelman (105) have been able to demonstrate the *in vivo* synthesis of bacterial RNA whose base composition is similar to the bulk DNA, using the organisms *E. coli* B and C-122, *P. aeruginosa* ATCC-10197, and *Bacillus megaterium* KM. This was done by means of a "step-down" transition which involves transferring the cells from a rich medium to a synthetic medium. In this process, RNA synthesis is apparently restricted to the type necessary for new protein synthesis. Like that synthesized after T2 infection, and unlike the bulk of the bacterial RNA, this new RNA had the same base analysis as the bacterial DNA. After heating and slow-cooling mixtures of this RNA with DNA from the same strain, complexes were detected by density banding. No interaction occurred, however, with heterologous heat-denatured DNA's. This fraction of RNA was therefore considered to carry information coded as in DNA. Gros *et al.* (95) have reported a similar RNA fraction in *Staphylococcus aureus*, and Ycas & Vincent (106), in yeast. If these are indeed messenger RNA's, then there arises the interesting implication that all the DNA of the bacterial cell is equally active, genetically. If this were not so, the rate of transfer of information from DNA to RNA would vary from locus to locus and the bulk messenger RNA would not correspond in base composition to the bulk DNA. Thus, one would not expect to find such a correspondence in higher organisms.

Bonner *et al.* (107) have isolated a complex containing DNA, protein, and newly formed RNA. Pea embryo chromatin was used as the enzyme in an *in vitro* synthetic system (108), which also included the four ribonucleoside triphosphates. The ATP was labeled with C^{14} . The labeled RNA remained bound to the deoxyribonucleoprotein; it was not susceptible to attack by RNase. Treatment of the DNA-RNA-protein complex with DNase or deproteinizing agents, however, rendered the RNA sensitive to RNase. Thus this complex behaves differently from the model complex of Schildkraut *et al.* (102). Furthermore, heating the complex to 60°C physically detached the RNA. Bonner *et al.* (107) concluded from the conditions of RNA release that protein denaturation, rather than the breakage of base-pair hydrogen bonds, was responsible. Undenatured DNA as well as RNA, in the ratio of 2 to 1, was released from the complex by heating.

In the past two years, several other groups have been concerned with the role of DNA itself in polyribonucleotide synthesizing systems. Weiss & Gladstone (109) noted a decrease in incorporation of cytidylate into ribonucleic acid on sonic treatment of liver homogenates. This led them to suspect the involvement of DNA. Steven (110) and Hurwitz *et al.* (111)

carried out similar studies with extracts from *E. coli*. The latter authors showed that DNA was indeed required for the enzymatic incorporation of nucleoside triphosphates into polyribonucleotides; they successfully used DNA from T2, *E. coli*, and calf thymus with the *coli* enzyme system. Using an extract from *Mycobacterium lysodeikticus*, Weiss & Nakamoto (112) also found a requirement for DNA. Moreover, they demonstrated (113) that the base analysis of the synthetic polyribonucleotide corresponded approximately to that of the DNA primer used, namely, *Pseudomonas*, *E. coli*, *Serratia*, or salmon sperm DNA. They have also presented evidence that the nearest neighbor frequencies of the product are similar to those of the primer (113a). DNA-dependent polyribonucleotide synthesis has also been investigated with enzyme systems from *Lactobacillus arabinosis* and *Azotobacter vinelandii* [Burma *et al.* (114); Ochoa *et al.* (115)]. In both systems, heat-denatured DNA was inactive as a primer, whereas native DNA from calf thymus, *L. arabinosis*, or *A. vinelandii* was active (114). This contrasts with the results of Furth, Hurwitz & Goldmann (116), who found that denatured DNA, Φ X-174 DNA, and polydeoxythymidylate, as well as native DNA, had primer activity in the *E. coli* system. The difference in primer requirement may arise from a difference in the enzymes, or, more probably, from a difference in their states of purity. Furth *et al.* (116, 117) also show that the base composition of the synthetic polyribonucleotide is complementary to that of the primer, which suggests specific base pairing as a template mechanism. Thirty-five per cent of their product is resistant to RNase, but becomes sensitive upon treatment with acid or heat (100°C) but not DNase. This suggests that the initial (e.g., double helical?) configuration of the product must be changed in order to render it sensitive to RNase; but the possibility of a hydrogen-bonded complex with DNA cannot be ruled out. Geiduschek, Weiss and Nakamoto (117a) have shown that complementary RNA synthesized in the presence of DNA primer does not remain attached to it. However, a complex can be formed by annealing RNA with its template DNA, but not with other DNA's. They further indicate that the primer DNA is not denatured by the polymerase system.

The DNA-dependent synthetic systems just discussed are presumably concerned with the synthesis of a minor fraction of the cellular RNA: that which transfers information. In this context it is interesting to note that the synthesis of genetic RNA without mediation of a DNA template has been demonstrated by Simon (118). Using Hela cells, he found that several different RNA viruses reproduced normally under conditions in which DNA could not be synthesized and the host DNA was abnormal. Furthermore, Loeb & Zinder (119) have discovered an RNA bacteriophage which can replicate in the absence of DNA synthesis.

The foregoing results make it seem most probable that an RNA-DNA complex of some kind exists as an intermediate in both *in vitro* and *in vivo* synthetic systems. Knowledge of its structure would provide a clue to the

mechanism of synthesis. One is therefore tempted to assume that the natural complex is the same as the artificial ones, previously discussed, but this is not necessarily true. For example, in annealing experiments DNA is capable of complexing with itself (85), as well as with RNA, but this is not a natural process. If there is, in fact, a single-stranded DNA template for RNA, then there must be mechanisms *in vivo* for untwisting the strands of native DNA and then for untwisting the product DNA-RNA strands and restoring the original partners. When one also considers that there is protein associated with the DNA, the problem in mechanics appears to be intractable. This must be all the more compounded in higher organisms, where the structure and continuity of the chromosome must be considered. It would therefore seem to be preferable for the transfer of information to occur without untwisting the DNA strands or removing the protein associated with the DNA. In such a scheme the protein might act as an enzyme or apoenzyme in the synthesis of both RNA and DNA. The observations of Bonner *et al.* (107) and Geiduschek *et al.* (117a) support this hypothesis.

REPLICATION

Notwithstanding the great progress that has been made in the field of DNA replication, a fundamental understanding of the process is just beginning. In this section we will discuss primarily some of the problems that have arisen out of current researches, and merely touch on the more abstract questions.

SPECIFICITY

A clearer understanding of the molecular biology of DNA can perhaps be achieved if the subject of specificity is considered from two points of view. Thus we shall consider the Watson-Crick (120) base-pairing hypothesis for the helical structure of DNA as distinct from their hypothesis for the replication of DNA (121). The fact that the latter also involves base-pairing has led to some confusion. The structural base-pairing hypothesis (120) together with the x-ray crystallographic analyses of Wilkins *et al.* (122, 123, 124) has solved nearly all of the problems relating to the helical structure. However, it has been pointed out by Donohue & Trueblood (125) that an adenine-guanine pair can probably satisfy the essential requirements of the helical structure, and this possibility must be kept in mind, particularly where noncomplementary base ratios are encountered. Base sequence is still a major unsolved problem, but this concerns function more than structure.

The Watson-Crick base-pairing scheme constitutes sufficient specificity, given a fixed helical structure, but it will not be sufficient during the act of replication if sugar-phosphate bonds are formed in regions which are not in a helical conformation. That is, an incoming nucleotide cannot find its proper position in the newly forming chain simply on the basis of a two-point contact, i.e., the hydrogen bonds. All four bases can satisfy this elementary requirement in several combinations, and indeed Donohue (126) and

Donohue & Trueblood (125) list many hydrogen-bonded pairs that are possible where the bases are not in a fixed configuration. In order to confer specificity so that one base may be distinguished from another, at least a third contact is required. Jehle (127) has suggested that specificity may be acquired through a multiplicity of forces, i.e., London-van der Waals forces, resulting in replica rather than complement (128) synthesis. That the Watson-Crick base-pairing is in fact a manifestation of specificity is not in question here. But in order to be able to decide among all the possible mechanisms of replication it will be necessary to understand the origin of the specific forces. Stated in other terms, the copying of a DNA molecule as in the Kornberg system (129), while quite remarkable, cannot be construed as proof of the base-pairing hypothesis but rather a necessary consequence of any mode of replication.

If the Watson-Crick hypothesis in fact describes replication, then the separated single strands must retain their helical configuration so that polarity of the chains and bond angles and distances can confer specificity. However, the untwisting of the chains means that the built-in specificity of the fixed structure is lost. The additional specificity which must now be sought elsewhere will, of course, result in but not arise from the pairing of bases. Specificity might be conferred by the protein associated with the DNA *in vivo*, or the DNA polymerase itself, in the sense that these might affect DNA chain configuration or the sequence of bases in the template. That is, in addition to the genetic information contained in a sequence (to be passed on, say, to RNA), the sequence might itself control the nature of neighboring sequences during the process of DNA replication. It may be significant that substitution of one base by an analogue with identical hydrogen-bonding properties does not occur equally at all available positions (130, 131); this would be the expected situation if each position on the template has some additional means of recognizing its proper occupant.

ΦX-174 DNA REPLICATION

The DNA isolated by Sinsheimer from the phage ΦX-174 (132) has non-complementary base ratios (133). Regardless of what the physical structure of this DNA may be, its replication presents still another disturbing problem for any mechanism which involves complementary base-pairing. Either the specific base-pairing hypothesis has to be abandoned, or another, highly specific event following DNA synthesis must be postulated. This problem also exists for other noncomplementary DNA's, such as that from blue-green algae (134).

It is not difficult to account for the structure of such molecules; Fresco & Alberts (135), for example, have shown that unpaired bases can loop out from a double helix without disturbing its regularity or spacing. Such a molecule would possess both helical and random coil character. But if a molecule of this type replicates semiconservatively by a complementary base-pairing mechanism, its base ratios become complementary; even if it

replicates conservatively, it will produce a mirror image of itself, so that the gross base analysis will become complementary, although the individual molecules are not. In order to conform with the observed base analysis, some of the DNA would have to be preferentially degraded subsequently or otherwise prevented from entering mature phage.

It has been reported by Sinsheimer (136) on the basis of the band density of DNA obtained on premature lysis, that Φ X-174 DNA goes through what appears to be a helical intermediate form during intracellular replication. It would be important from the standpoint of both structure and replication mechanism to know whether this form possesses complementary base ratios. Both this intermediate form (136) and mature Φ X-174 DNA are able to infect protoplasts (137, 138), so apparently replication can be initiated at either stage. The ultraviolet spectra investigations of Setlow & Setlow (139, 140, 141) also indicate that intracellular Φ X-174 DNA, as well as T2 DNA, goes through ordered and disordered states during replication.

In the Kornberg system, Josse (142) has reported that Φ X-174 DNA used as primer in the presence of labeled nucleoside triphosphates goes through a hybrid stage in which the proportions of bases incorporated are complementary to those of Φ X-174 DNA, and which possesses the properties of a double helix. Since synthesis can be continued manifold beyond this stage, it seems that both the original and the helical forms are capable of template action [as Kit (143) has pointed out]. The product, however, is always found in the complementary, helical state and the synthetic system must therefore be unlike the intracellular one for Φ X-174 in at least one respect.

SEMICONSERVATIVE REPLICATION

The experiment of Meselson & Stahl (144) showed clearly that *E. coli* DNA replicates semiconservatively. This has been confirmed by Sueoka (145) using *chlamydomonas* and by Simon (146) using Hela cells. This means that each daughter cell receives two units, one old and one newly synthesized. The nature of these experiments did not permit a more explicit molecular interpretation. The experiments of Cavalieri and Rosenberg (69, 147, 148, 149), however, showed that the conserved unit was the double helix and that each daughter cell, therefore, receives one old and one newly synthesized double helix, joined together as a pair. Replication, therefore, does not involve strand separation. The mechanism may well be similar to that occurring when messenger RNA is synthesized on an undenatured DNA template.

The pair of double helices has been called a biunial molecule. The fact that biunial molecules are four-stranded was shown by enzyme kinetics (69), x-ray kinetics (149), and electron microscopy [Hall & Cavalieri (150)]. In the first two methods, the decrease in molecular weight, determined by light scattering, was related to the number of single chain scissions and from this the number of strands per molecule was calculated. In the

third method, the lengths of molecules were measured in the micrographs in order to calculate the ratio of mass (from light scattering) to length. The value obtained for pneumococcus DNA, which is biunial, was close to that expected for four-stranded molecules. Furthermore, the shadow height indicated that the two double helices were not intertwined but rather lay side by side. The nature of the bonds (biunial bonds) holding the two helices together remains to be elucidated but the weakness of the linkage indicates that they are probably few in number.

The two-stranded, conserved unit of *E. coli* DNA has been isolated from a synchronized culture which was about to begin DNA synthesis (148). The DNA at all other times was biunial, as found in random cultures. Two-stranded DNA molecules also appear to be characteristic of nonproliferating tissues; such DNA has been called unitary (147). The DNA of these cells is undoubtedly also semiconserved, but the units remain separated in the absence of replication.

Taylor, Woods & Hughes (151) have shown that replication is also semi-conservative at the chromosomal level. Their results with *Vicia faba* have been extended to other plants and to animal cells in tissue culture (152, 153, 154), and to *E. coli* 15T- [Forro & Wertheimer (155)]. Since both chromosomes and the DNA molecules composing them replicate semiconservatively, the anaphase chromosome must be double and each conserved half must contain one conserved unit (or double helix) of each DNA molecule.

UNWINDING DNA

One of the great obstacles in the Watson-Crick replication hypothesis is the necessity for having to untwist the two chains. A number of very clever schemes have been devised to achieve this (e.g., 156) but these will not be discussed here. Recent calculations on the time of unwinding have been made by Kuhn (73, 74) and Longuet-Higgins & Zimm (72). Depending on the model chosen, the outer limits are 1.4 seconds and 150 days for a 10 million-molecular weight DNA molecule. The authors tend to favor the short time, admitting that the assumptions made are the best possible to reduce the unwinding time to a minimum. For a 120 million-molecular weight molecule, the equation of Longuet-Higgins & Zimm leads to a value of 12.4 minutes for the minimum unwinding time. This length of time is longer than that observed for a single replication of T2 DNA. If the T2 phage genome is a single DNA molecule (see page 258), untwisting is ruled out unless one assumes that the T2 DNA unwinds along many segments simultaneously. Such a mechanism appears unlikely, for it would involve the formation and repair of a series of breaks, as well as a synchronized system of unwinding in order to allow all newly synthesized pieces to be joined into a large molecule without any errors. At the higher, chromosomal level where not one molecule is involved, but thousands, the problem of untwisting old from new strands while preserving their relationships within the chromosome is perhaps

intractable. The same mechanical problem would exist if the conserved units, the double helices, were twisted around each other. The observations of Hall & Cavalieri (150), however, indicate that they are not.

In view of the conservation of the double helix, it is perhaps most reasonable to suppose that its replication takes place without any intermediate separation of strands. A mechanism for this has been proposed [Bloch (157)] in which the hydrogen bonds break and the bases rotate so that they can act as templates in the wide groove of the double helix. The newly synthesized helix, however, is intertwined about the old one. By modifying the scheme somewhat it has been possible to show, using models, that a new helix can be formed so that it will eventually be found alongside the old one and attached to it by biunial bonds (158).

THE KORNBERG SYNTHETIC SYSTEM

The Kornberg polymerase has had striking success in copying a large number of DNA primers (129). The fact that the DNA's used have had a widely varied base composition indicates that in general the polymerase is directed by the primer DNA. How does the relatively small enzyme molecule manage to synthesize large DNA molecules so accurately, while free in solution? There are a number of other unsolved questions concerning the *in vitro* system which will be discussed in the following paragraphs.

Lack of biological activity in the product.—Up to the present, no DNA with biological activity has been synthesized. Attempts have been made using *Haemophilus influenzae* DNA as primer (162), but no transforming activity was observed. This was attributed to the presence of contaminating nucleases. Since it is possible to achieve a twentyfold net synthesis of DNA (162), one would expect at least a small amount of activity on the grounds that nucleases should attack the product at random and it is improbable that all active sites in all molecules would be destroyed. There are at least two other possible causes for the lack of activity.

The first concerns bias in base sequences, which may be introduced by the polymerase. In the unprimed reaction using all four deoxynucleoside triphosphates as substrates, the resulting d-AT copolymer has a regularly alternating sequence of adenine and thymine in each chain (88). When the substrates are d-GTP and d-CTP, one chain contains only deoxyguanylate and the other only deoxycytidylate (102). The polymerase, therefore, introduces complete bias in both instances; in the first case an adenylate must follow a thymidylate and vice versa; in the second case, a guanylate must follow a guanylate and a cytidylate a cytidylate. It is altogether possible then that some bias is introduced even in the presence of primer; it is obvious what this would do to the information content of the product DNA. Although the nearest neighbor pattern of the synthetic DNA is quite similar to that of the primer, an error of 1000 nucleotide sequences out of 10^6 nearest neighbors could not be detected by this method, as Josse *et al.* (129) have stated.

A second possibility for the lack of biological activity might be blank

spots in the product. Thus, it is possible, particularly when using denatured DNA as the primer, that the DNA loops out, thereby losing contact with the enzyme for, say, five or ten nucleotides; these missing five or ten residues in the product would constitute a blank spot in that particular region of the DNA. Blank spots could also arise from another source. Since more than one polymerase molecule may eventually be used for the synthesis of a single DNA molecule, errors might develop in regions of the product where one polymerase molecule ends and another begins.

Primer requirements.—Whether or not native or denatured DNA functions more efficiently as a primer appears to depend on the polymerase preparation. With the most highly purified preparations of polymerase from *E. coli*, native DNA is utilized more efficiently than heat-denatured DNA (163), whereas in earlier work denatured primer had resulted in a higher rate of synthesis (159). However, purified preparations of polymerase from T2-infected cells prefer heat-denatured DNA as primer (163). The known polymerases from mammalian sources appear to require denatured DNA exclusively (164). Limited DNase action on native DNA increases its priming ability (159); this is probably due to the creation of ends where DNA synthesis can begin. This suggests that the difference between systems requiring native and denatured DNA as primers may be due to the presence of nuclease impurities which act preferentially on denatured regions.

Rates and mechanism of in vitro synthesis.—It has been shown by sedimentation experiments [Schachman *et al.* (88)] that the unprimed synthesis of the d-AT copolymer molecule must be very rapid, since the polymerizing reaction mixture at no time contains anything but high molecular weight ($\approx 4 \times 10^6$) polymer together with fragments having a molecular weight of about 10^3 or less. It would be interesting to know how many polymerase molecules were present in order to calculate the rate of synthesis of each DNA molecule from the low molecular weight precursors.

Schachman *et al.* also found that d-AT-primed reactions are more rapid in over-all rate than DNA-primed ones, in addition to lacking the long lag characteristic of unprimed reactions giving the same product. However, in contrast to the unprimed reaction, there appears to be a gradual synthesis of intermediates [Baldwin *et al.* (165)]. Baldwin and co-workers used poly d-AT as primer with d-ATP and 5-bromodeoxyuridine triphosphate (a heavy analogue of thymidine triphosphate) as substrates. Since the primer was completely double-helical under the conditions of synthesis, they infer that if there is any hydrogen bond breakage or strand separation it must be concurrent with synthesis. They did find material of density intermediate between those of the primer and the eventual product, poly(deoxyadenylate+polybromodeoxyuridylate) in the early stages of the reaction; but there was no discrete hybrid band. The melting point of this complex material was also intermediate. When it was held above its melting point, a very slow (~ 12 hours) separation into heavy and light macromolecular components took place. If this was strand separation, it was extremely slow in

spite of the low molecular weight of the material and its probable concomitant degradation.

Josse, Kaiser & Kornberg (129) have proven, in nearest neighbor analyses of synthetic DNA made with various primers, that the two strands of the double helix must have opposite polarity. This is also required by the dimensions and pairing of the Watson-Crick model (120). However, structural polarity does not determine the physical direction of synthesis; i.e., it is possible for a chain to grow in either direction along its template, if indeed synthesis is sequential.

THE BACTERIOPHAGE CHROMOSOME

It has been pointed out above that the DNA of T2, and by inference T4, probably exists as a single, discrete entity (1, 2, 3). The same has been found for the DNA of smaller phages (132). What is the nature of this entity, which may be called the phage chromosome? From a purely genetic point of view a single DNA molecule is identical with a linear array of DNA molecules held together by protein links, provided there is specificity in the end-to-end aggregation. However, physical and chemical means can be used to distinguish the two possibilities.

In a recent paper, Kozinski (166) infected N^{15} -labeled *E. coli* in N^{15} medium with light, P^{32} -labeled T4 phage. After premature lysis, he banded the DNA and observed increasing amounts of P^{32} in the heavy (N^{15}) band, whose density appeared unaltered in spite of the presence of light, parental DNA therein. The parental moieties therefore comprised less than about 10 per cent of any DNA particle. After mature lysis all the P^{32} was in the heavy band, and no DNA of hybrid density was observable. However, after sonication of the DNA all the P^{32} appeared in a band of hybrid density, which had the same width as the heavy band. Kozinski suggested that the sonicate might consist of subunit molecules which are joined end-to-end to form the larger unit originally isolated. Replication appears to be semi-conservative at this level.

In a later paper Kozinski & Uchida (167) confirm these findings using a different approach. By introducing P^{32} phosphate into an infected culture at various times they were able to show that phage-precursor DNA which is formed at different points in time nevertheless finds its way into a single phage particle. These findings would be consistent with independent replication of the subunit molecules, which would then assemble in some specific way to form the viral genome at maturation; or the dispersal of the subunits could be due to physical cross-over, perhaps at the points of joining of the DNA molecules in the chromosome.

The existence of physical cross-over is indicated by the experiments of Meselson & Weigle (168). These authors crossed two genetically labeled mutants of λ phage, one of which was labeled with the density marker $C^{13}N^{15}$. The progeny phage were banded and the density region between the two parental types was assayed genetically. Two discrete regions containing

only the two types of recombinants were found. These recombinants were of necessity composed in part of DNA from the heavy parent, which must have recombined physically with light DNA containing the other genetic marker.

Single infection with heavy λ phage produced only hybrid and light progeny, from which Meselson & Weigle (1968) concluded that the λ chromosome consists of two conserved units which must separate in order to replicate. In λ phage, unlike T4, the chromosome as a whole appears semiconserved because there is less than 15 per cent recombination to disperse the DNA. Since recombinants were found containing more than 50 per cent of one parental DNA they concluded that recombination by chromosome breakage can occur without separation of the two conserved subunits of the parent chromosome and, therefore, that the phage chromosome need not replicate in order to recombine. Perhaps an explanation for heterozygotes might be found in a rare event involving only one of the two units.

Kellenberger *et al.* (1969) also present evidence for physical exchange of DNA in recombination. Two mutants of λ phage having different densities were used. These density markers behave in crosses, as does any other genetic marker, and they could be identified by banding the whole phages in cesium chloride. One of the parental phages was labeled with P^{32} . Recombinants of intermediate density formed separate bands which were found to contain P^{32} . Random dispersal of the parental DNA was ruled out.

Both these studies (1968, 1969) show that distance on the genetic map is approximately proportional to physical length along the chromosome. However, Meselson & Weigle (1968) suggest that some more complex situation, such as favored break points, may be required by their data. These might occur between subunits such as those found by Kozinski.

BACTERIAL TRANSFORMATION

Recent work indicates that bacterial transformation may also involve physical incorporation rather than copying of the transforming DNA. Lacks & Hotchkiss (1970) transformed pneumococcus to the ability to synthesize amyloamylase, and found that production of the enzyme reached its maximum rate in ten minutes, well before cell division and, in all probability, before DNA synthesis. The rate was proportional to the extent of permanent genetic change, thereby ruling out any abortive activity of the transforming factor. They confirmed that recombination occurred before enzyme production by repeating the same observations with transforming DNA possessing a genetic defect in the same cistron as the defect in the recipient cell; here, recombination was necessary to reconstitute an active cistron. Unless in these experiments only those cells (at most a small percentage of the total) were transformed which were in the process of replicating their DNA, physical breakage and rejoining is required. Voll & Goodgal (1971), using *Hemophilus*, have presented a different type of evidence leading to the same conclusion. They transformed the cells in saline, in which no cell growth was possible, using transforming DNA containing a marker linked to another

in the recipient cells. The cells were lysed and the DNA isolated at various intervals after exposure to the transforming factor. The isolated DNA was assayed for linkage between the two markers by using it to transform cells possessing neither marker. Linkage began to appear at once, and reached half its maximum in 15 minutes. Since there was no discernible DNA synthesis at this time, physical breakage and recombination is strongly implied.

Fox and Hotchkiss (172, 173) have examined the fate of DNA after it is irreversibly fixed by bacteria. After short periods of incubation, there is no physical or biological dilution of the transforming DNA. Furthermore, DNA reisolated immediately following fixation is unable to cause transformation; i.e., there is a loss of function which is, however, recovered with longer incubation (173). In this same short period, it has also been shown (173) that genetic linkage can take place. Since it is believed that little or no DNA synthesis occurs during this early period, physical breakage of the DNA is again implied.

Although copy-choice (174) cannot be ruled out as an additional mechanism, physical cross-over is sufficient to account for recombination in bacteriophage and for phenomena such as transformation and transduction as well. There is also reason to reject copy-choice as the mechanism of non-reciprocal recombination in higher organisms. The discovery of episomal transduction (175) and the existence of episome-like factors in higher organisms (176) raise the possibility that intracellular transduction might be involved in "gene conversion." Thus, it is quite conceivable that replication and recombination are in all cases independent processes. This would simplify the problem of the replication mechanism and eliminate the necessity for much complex entanglement and disentanglement.

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METABOLISM OF NUCLEIC ACIDS^{1,2}

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INTRODUCTION

In order to avoid extensive overlapping with the accompanying reviews by Grunberg-Manago (1), and by Simpson (2) which deal with the mechanisms available for the biosynthesis of nucleic acids and with the relationships of the nucleic acids to protein biosynthesis, this article deals primarily with information concerning the metabolic interactions of nucleic acids that has been derived from experiments with intact cells.

RNA SYNTHESIS AS A FUNCTION OF GROWTH RATE

In this review, wherever it has been possible to distinguish the metabolism of one ribonucleic acid (RNA) fraction from the other (nuclear RNA, ribosomal RNA, etc.), the differentiation is emphasized; otherwise, the discussion pertains to RNA in general.

In studying *Aerobacter aerogenes* at various stages of growth, Dean & Hinshelwood (3) have found that the rate of increase in RNA is dependent on the growth rate, and that the ratio of RNA to deoxyribonucleic acid (DNA) increases as that rate increases. Neidhardt & Magasanik (4), using *Escherichia coli*, have studied the variations in the content of RNA and protein that occur with changes in the growth rate and have found that the ratio of RNA to protein, when plotted against the growth rate, does not yield a linear relationship. At very low growth rates this ratio remains approximately constant, while at high growth rates the ratio increases, with the increase being attributable primarily to the increase in RNA. When the growth rate is artificially decreased, a corresponding cessation of RNA synthesis occurs, while the synthesis of protein and DNA continues. On the other hand, when the growth rate is increased, an acceleration of RNA synthesis takes place, while the rate of formation of protein and DNA remains constant and only increases at a later time. These authors believe that, during accelerated growth, the rate of protein synthesis is restricted by a limitation on the availability of RNA templates, despite the presence of an adequate supply of the necessary precursors of proteins. In contrast, when the growth rate is decreased, the number of ribosomal RNA templates that are available is no

¹ The survey of the literature pertaining to this review was completed in November 1961.

² The following abbreviations will be used: MP, DP, TP (mono-, di-, and triphosphate, respectively); A, C, G, I, T, U, X (adenosine-, cytidine-, guanosine-, inosine-, thymine-, uridine-, and xanthosine-, respectively, when combined with the foregoing phosphate designations); d (deoxy, when used as a prefix); ATPase (adenosine triphosphatase); DNase (deoxyribonuclease); RNase (ribonuclease).

longer limiting, but the limiting factor becomes, rather, the availability of protein precursors. These workers have also found that the amount of soluble RNA (not sedimentable at 100,000 g) is at all times a constant proportion of the total RNA. Kjeldgaard (5), who studied the relationship between the synthesis of RNA and protein in *Salmonella typhimurium*, comes to similar conclusions. In addition, his results indicate that a proportionality exists between the number of ribosomes contained in the cell and the rate of protein synthesis.

The experiments of Hayashi & Spiegelman are in accord with these general observations (6). These investigators have studied the type of RNA synthesis which occurs in bacteria when their growth rate is abruptly decreased. This type of synthesis consists of the preferential synthesis of an RNA ("messenger" or "informational") which is heterogeneous in size, metabolically unstable, and exhibits a base ratio analogous to that of the cellular DNA (6). The preferential synthesis of such RNA under these conditions is consistent with the continuing synthesis of protein which occurs in these cells and with the postulated role of such RNA as an intermediary between DNA and the protein-synthesizing apparatus (1, 2).

METABOLIC STABILITY OF RNA

Mandelstam & Halvorson (7) find that, although negligible breakdown of either protein or RNA occurs during the growing phase of *E. coli*, the protein of nongrowing cells shows a turnover of 5 per cent per hour (i.e., degraded and resynthesized) while maintaining the total amount of protein at a constant level. Although the ribosomal RNA is degraded at the same rate, it is resynthesized at a rate of only 1.7 per cent per hour. Consequently, the ribosomes which were resynthesized under conditions of starvation have a higher ratio of protein to RNA. In contrast to the behavior of this ribosomal RNA, the soluble cytoplasmic RNA shows a negligible rate of breakdown and a high rate of synthesis. Accordingly, the net effect appears to be a transfer of ribosomal RNA to the nonparticulate cytoplasmic fraction. These authors also conclude that during starvation the ribosomes act as a reservoir which supplies one-half of the amino acids and all of the ribonucleotides passing through the free pools. Higher rates of turnover of protein in growing *E. coli* (2.87 per cent per hour) than those previously described have been reported by Fox & Brown (8). Goldstein & Brown (9) describe higher rates of turnover of protein in nongrowing *E. coli* (10 per cent per hour), and also emphasize the lack of net synthesis of RNA in *E. coli* under conditions of protein starvation, despite the availability of RNA precursors in the medium, and despite the concurrent availability of amino acids from the turnover of protein. It is suggested that either protein turnover does not yield amino acids in a utilizable form, or RNA and protein turnover are so tightly coupled that all protein degradation leads to a concomitant depolymerization of RNA and thus to no net synthesis of RNA.

Feinendegen *et al.* (10) have found that under conditions of active

growth the RNA of HeLa cells does not show any extensive turnover. Graham & Siminovitch (11) have labeled Earle cells with P^{32} -phosphate and subsequently allowed them to grow in a medium containing nonradioactive phosphate. They find that an initial loss of P^{32} -RNA occurs (10 to 30 per cent of the total label) which lasts for 24 to 48 hours. Subsequently, however, the P^{32} -RNA is maintained at a constant level.

Since the evaluation of the metabolic stability of RNA *in vivo* often involves the measurement of the rate of labeling of RNA or of the rate of loss of label from DNA, the long period necessary for pool equilibration can seriously affect the results. This has been emphasized by Watts & Harris (12), whose experiments show that in macrophages the P^{32} label in RNA continues to increase when the cells are placed in a P^{31} -phosphate medium; in contrast, C^{14} -adenine in the RNA is lost by subsequent growth in a medium containing nonradioactive adenosine. Using *Chlorella* cells, Miyachi & Tamiya (13) suggest that the phosphate used for the synthesis of RNA is derived from a different pool than that used for the synthesis of DNA and of phosphoprotein. The experiments of Feinendegen *et al.* (10) referred to previously indicate that when HeLa cells are incubated with radioactive thymidine, and subsequently transferred to a nonradioactive medium, all further incorporation of radioactivity into DNA ceases. In contrast, similar experiments performed with radioactive ribonucleotide precursors (10, 14) emphasize the continuing extensive incorporation of radioactivity into both the RNA and the DNA when the cells are subsequently placed in the nonradioactive medium. Experiments performed with nuclei isolated from pea seedlings (15) also demonstrate the difficulty in inhibiting the continuing incorporation of radioactivity into RNA by the method of isotopic dilution. This phenomenon is therefore not due to the presence of the cytoplasmic pool alone. The results of Allfrey & Mirsky (16) emphasize that ribonucleotides incorporated *in vitro* into the RNA of calf-thymus nuclei show a differential lability: the label from adenosine, once incorporated, is relatively stable, while that incorporated from uridine, and to a lesser extent from P^{32} -phosphate, is very unstable and a high proportion is lost upon subsequent incubation in a nonradioactive medium. An additional difficulty in the interpretation of such experiments is the inability to distinguish between lack of turnover and a turnover in which the products of breakdown are efficiently re-utilized for synthetic purposes. (For further discussion on nucleotide pools see page 282.)

In order to circumvent these difficulties, Davern & Meselson (17) have used another method in an attempt to resolve the question of the metabolic stability of ribosomal RNA of actively growing *E. coli*. They have performed density gradient experiments with the ribosomal RNA of *E. coli* B grown initially in a medium containing N^{14} - and C^{13} -compounds and subsequently, for several generations, in the presence of a medium enriched with N^{14} - and C^{12} -compounds. These authors find that under these conditions only a heavy ribosomal RNA (N^{15} - C^{13}) and a light ribosomal RNA (N^{14} - C^{12}) could be

distinguished. No appreciable decrease in the amount of heavy RNA occurred in the first three generations of growth in the $N^{14}\text{-C}^{12}$ medium and no intermediate species of RNA corresponding to $N^{15}\text{-C}^{13}$ plus $N^{14}\text{-C}^{12}$ was observed. If the heavy ribosomal RNA which had been synthesized in the $N^{15}\text{-C}^{13}$ medium were being actively turned over then it would be expected to decrease in amount when the *E. coli* were subsequently grown in the $N^{14}\text{-C}^{12}$ medium. Alternatively, if the products of metabolism of heavy RNA were extensively and efficiently utilized for the synthesis of the $N^{14}\text{-C}^{12}$ RNA, then the new ribosomal RNA should contain both types of molecules ($N^{15}\text{-C}^{13}$ and $N^{14}\text{-C}^{12}$) and, therefore, should have an intermediate density. Accordingly, it appears that under the conditions of these experiments in which enhanced synthesis of ribosomal RNA occurs (cf. 3) no exchange reactions of ribosomal RNA with precursors or of ribosomal RNA molecules among themselves takes place. The metabolic instability of a small fraction of the cellular RNA ("messenger") and its possible relationship to other cellular reactions is discussed by Grunberg-Manago in the accompanying article (1).

HETEROGENEITY OF RNA

In metazoan cells the amount of RNA present in the nucleolus and in the nucleus is very small and is species-dependent. By micromanipulation techniques, Edstrom (18) has determined that the nucleoli of individual spider oöcytes contain 0.3 per cent of the total cellular RNA, while the nucleoplasm contains 1.4 per cent. In ungerminated pea embryos, Stern *et al.* (19) find that the nucleolar RNA is 1.6 per cent, while the total nuclear RNA is 8 per cent of the RNA of the embryonic tissue.

Lane & Allen (20) have found that only about 40 per cent of the total RNA of wheat germ can be extracted with phenol. Of this, 10 per cent corresponds to a low molecular weight RNA which, upon alkaline hydrolysis, yields a high proportion of adenosine and also a high proportion of guanosine diphosphate. The remaining 30 per cent of the extractable RNA is a high molecular weight RNA which, upon alkaline hydrolysis, yields equivalent proportions of the four ribonucleosides and again a high proportion of guanosine diphosphate. The high guanosine diphosphate is reminiscent of the end-group analysis of S-RNA (21 to 23). The portion of this RNA which is not extractable by phenol appears to be associated with the nuclei (24). The difficulty in phenol extraction of the nuclear RNA of different tissues (calf thymus, rat spleen, and kidney) also has been demonstrated by Gregoriev & Mant'eva (25). Thus, after administration of P^{32} -phosphate, this residual nuclear RNA has 40 to 80 times the specific activity of the cytoplasmic RNA. Using a phenol-Duponol procedure, however, Philipson (26) obtains a quantitative recovery of RNA and of DNA from HeLa cells.

Yamana & Sibatani (27), after the incorporation of P^{32} -phosphate, have extracted homogenates of rabbit lymphocyte cells and of rat livers with phenol. The extractable RNA has a high content of guanylic acid and a low specific activity. In the residual RNA, the content of guanylic acid is low and

the specific activity is high. These workers suggest that the extractable RNA is duplicated only once in the mitotic cycle, while the residual RNA may be turned over rapidly. Heterogeneous labeling of RNA in rat liver homogenates also has been described by Schneider & Potter (28). Reid (29) has studied the rate of labeling of the RNA of various microsomal fractions from rat liver, as well as their nucleotide composition. His results indicate that a disproportionality in labeling occurs in these fractions, as well as a variability in their nucleotide composition.

Roots of pea seedlings when extracted with cold perchloric acid (1 *N*) display a residual nonextractable RNA with a base composition which differs from that of the RNA which is extractable by the perchloric acid (30). Finamore & Volkin (31) describe the presence of two RNA fractions in the mature ovaries of *Rana catesbiana*: one of these is soluble in 0.5 *N* perchloric acid, while the other is not. These two RNA fractions differ in their base composition.

Kit (32) has studied the nucleotide composition of the microsomal fraction and of two nuclear fractions (extractable and nonextractable with phosphate) of various tissues. These findings emphasize the similarity which exists in the base composition of the RNA of the various fractions within a tissue, as well as between the tissues. However, such results are not in agreement with others in the literature and the differences are discussed by the author.

A change in the composition of the RNA of the chloroplasts of *Euglena* cells, which coincides with the time of synthesis of chlorophyll, has been reported by Brawerman *et al.* (33). Santer *et al.* (34) find that the base composition of the ribosomal RNA of *E. coli* varies at different stages of growth, and that the type of growth medium used can also affect the base composition of the ribosomal RNA (cf. 5). In the early stages of growth, the base composition of the ribosomal RNA appears to be similar to that of the DNA, while the base composition of the soluble RNA appears to remain constant under these various conditions.

Brown (35) has found that the sedimentation coefficient of high-molecular-weight RNA decreases in the presence of L- and of D-amino acids, giving a high proportion of products of 3S size. It is suggested that all high molecular weight RNA can be dissociated to 3S subunits. These observations are related to those of Otaka *et al.* (36), which indicate that the incubation of the ribosomal RNA of yeast with trypsin lowers the sedimentation coefficient of the RNA from 20S to 4S. After attaining the value of 4S, the sedimentation coefficient remains constant even if large amounts of trypsin are added. Similar decreases in the molecular weight of RNA upon incubation with nonenzymatic proteins have been described (37). At present, it is difficult to interpret such findings, especially because of the ubiquitous presence of ribonuclease (RNase) (38), but the results are similar to those obtained with the deoxyribonuclease (DNase) of erythrocytes on DNA and described under nucleases.

In contrast to this depolymerization of RNA is the polymerizing effect of polyalkylamines on RNA. Yoshikawa & Maruo (39) have provided provisional identification of a polyalkylamine or an alkylidiamine from *B. subtilis* which, when added to RNA *in vitro*, causes association of the RNA and the formation of large macromolecules. When these compounds are added to cultures of *B. subtilis* they increase the production of exoenzymes, as well as the turnover of RNA and polyphosphates. The presence of polyalkylamines in ribosomes (40) and the ability of such compounds to stabilize ribosomes (41) are suggestive of the biological function of these compounds.

These results tend to emphasize the heterogeneity inherent in cellular RNA. The heterogeneous composition of S-RNA, despite its gross appearance of homogeneity, is well established (42, 43) and the "informational" or "messenger" RNA also appears to show a heterogeneity in molecular weight (5). By implication, and based in part on the preceding discussion, it may be assumed that the RNA extractable from various cell fractions probably consists of a similarly heterogeneous population of molecules. This could be related in part to the heterogeneity inherent in the ribosomal proteins. Waller & Harris (44) have found that in the ribosomes of *E. coli* the proteins are basic and show two major NH_2 -end-groups (methionine and leucine), with an average molecular weight of 25,000; yet, within each class, as defined by end-groups, not all chains are identical. These authors have also emphasized the similarity between these proteins and the histones of calf thymus: the latter are composed of a large number of proteins, yet alanine and proline account for 90 per cent of the end groups.

SITES OF RNA SYNTHESIS

A number of ingenious approaches have been used to define the sites of synthesis of RNA. Nevertheless, the limitations of the available techniques and the complexity of metabolism make it natural that the interpretation of the results in this field should show subjective variations.

Using a technique which permits the localization of radioactivity in very thin sections of *E. coli*, Caro & Forro (45) have concluded that a large fraction of the RNA is synthesized in or near the nucleus and is subsequently transferred to the cytoplasm. The association of RNA with the chromatin bodies of *Bacillus megatherium* KM has been studied by Ezekiel (46). Using cells which are inhibited with chloramphenicol, most of the RNA synthesized under these conditions appears to be localized in the chromatin bodies, while the formation of RNA in the cytoplasmic fraction is inhibited. The RNA cannot be removed from the chromatin bodies after treatment with DNase or with lipase. In the presence of chloramphenicol some of the cytoplasmic RNA also appears to migrate to the chromatin bodies. An association of newly synthesized RNA with the chromatin of pea embryos has been demonstrated by Bonner *et al.* (47); this RNA is not susceptible to the action of RNase unless the chromatin is pretreated with DNase or is heated to 60°.

Results of radioautographic experiments on protozoan and metazoan

cells also lead to the conclusion that the nucleus becomes extensively labeled early during the incubation and that the label appears to move from the nucleus to the cytoplasm, a circumstance suggestive of a precursor-product relationship (48). Nevertheless, the available evidence is not without discrepancies, and areas of relative ignorance remain for further investigation. McMaster-Kaye (49) has studied this problem, using the salivary glands of *Drosophila* at various periods of growth. The findings indicate that the incorporation of precursors into the nucleolar RNA is independent of the growth rate and that complete turnover of the nucleolar RNA occurs within one hour or less. This is attributed to a degradation of the RNA in the nucleolus rather than to a movement of the RNA molecules from the nucleolus to other cell compartments. In contrast, the cytoplasmic RNA shows no evidence of turnover. The chromosomal RNA appears to occupy an intermediate position; it reaches a lower specific activity than does the nucleolar RNA, but higher than that of the cytoplasm, and shows evidence of the turnover of RNA. It is concluded that (a) the RNA of the nucleolus is synthesized in that entity, (b) it has a unique function which is accomplished by degradation of the molecule, and (c) the products of degradation may be useful in some other function. In addition, based on the long delay in the acquisition of labeling by the non-nucleolar RNA which is observed in these experiments, it is suggested that the nucleolar RNA and the non-nucleolar RNA have different ultimate precursors.

Attempts to differentiate between the various parts of the nucleus as sites of RNA synthesis have led to conflicting evidence. The experimental evidence indicates that the site of synthesis of the nuclear RNA is either in the chromatin or in the nucleolus, or in both. The cytoplasmic RNA is considered to be derived either from the chromatin and the nucleolus, or from the chromatin after passing through the nucleolus, or from the nucleolus after passing through the chromatin. [For cumulative references, see (47, 50).] An attempt by Rho & Bonner (15) to study this interrelationship in isolated nuclei of pea seedlings emphasizes the complexity inherent in the problem because of the intricate kinetics involved. The results do not provide an unequivocal resolution of the problem and could be interpreted to indicate that the synthesis of RNA occurs independently in the chromatin and in the nucleolus.

A different experimental approach has been to divide cells into two fractions, the nucleated and the enucleated portion, in order to measure the ability of each fraction to synthesize RNA. Goldstein *et al.* (51), using enucleated pieces of cytoplasm from human amnion cells, could not demonstrate an incorporation of either purines or pyrimidines into RNA under conditions which permitted the incorporation of amino acids into proteins. Nevertheless, the authors regard the problem of the site of RNA synthesis as still unresolved, since Harris (52) has provided evidence, based on radioautographic data, which suggests that the cytoplasmic RNA is not derived from nuclear RNA; however, the latter results have been contradicted by Taylor

(53). Using *Acetabularia*, Richter (54) has come to the conclusion that the enucleated portion of the cell can maintain and probably differentiate RNA, but cannot synthesize net amounts of RNA. The ability of the enucleated portion of *Acetabularia* to maintain the synthesis or turnover of RNA has been substantiated by findings of Sutter *et al.* (55). These show substantial incorporation of P^{32} -phosphate and of C^{14} -adenine into the RNA. A refinement of this problem has been introduced by Schweiger & Bremer (56), who find that the ability of the cytoplasm of *Acetabularia* to synthesize RNA is light-dependent. If a period of darkness precedes the enucleation process, the cytoplasm can synthesize RNA upon exposure to light. In contrast, however, the synthesis of RNA does not occur in the cytoplasmic fraction if the *Acetabularia* are placed in light prior to enucleation. Since the *Acetabularia* cannot synthesize RNA in the absence of light, these workers (56) conclude that the nucleus liberates compounds into the cytoplasm which accumulate in the absence of light. Subsequently, when the cells are enucleated and exposed to light, such accumulated compounds appear to permit the maintenance of synthesis of cytoplasmic RNA.

Perry *et al.* (57) have approached this problem by a different method: they irradiate various portions of the nucleus with a microbeam of ultraviolet light and study the effects on the subsequent incorporation of cytidine. Their results led them to the conclusion that two-thirds of the incorporation into the RNA of the cytoplasm is dependent on the nucleolus and that one-third of the incorporation into the extra-nucleolar parts of the nucleus also is dependent on the nucleolus. Comparable results have been obtained by Seed (58) using microbeam x-ray irradiation. Using a similar approach with *Acetabularia*, Olszewska *et al.* (59) reach the conclusion that the nucleus controls the synthesis of both RNA and protein.

The preceding discussion emphasizes the dependence of RNA synthesis upon a functional chromatin body or nucleus, which in itself accounts for a small proportion of the total RNA. To date, however, no experiment provides definitive proof that the nucleus is the source of intact cytoplasmic RNA, although by inference it may be assumed that the DNA-dependent synthesis of RNA, catalyzed by RNA polymerase (1), occurs in the proximity of the nucleus. There is as yet no evidence that DNA may provide the code for the synthesis of all the cytoplasmic RNA; e.g., the presence of odd methylated bases in S-RNA makes it difficult to visualize DNA as providing a ribonucleotide sequence unless correspondingly odd bases can be found in DNA. However, an alternative interpretation could be that S-RNA is first synthesized from a DNA matrix without the methyl groups and that it is subsequently methylated in the appropriate positions in the presence of methionine (cf. page 289).

DNA REPLICATION *IN VIVO*

In protozoan and metazoan cells.—Simmel & Karnofsky (60) have shown that no DNA synthesis occurs in the unfertilized sand dollar egg and sperm.

Shortly after fertilization, however, and prior to fusion of male and female pronuclei, the synthesis of DNA is initiated in both the male and the female pronuclei. During this stage an appreciable amount of DNA synthesis occurs in the pronuclei and continues after fusion has occurred. Thus, in this biological system, fertilization appears to be associated with a cytoplasmic change which initiates the prompt onset of DNA synthesis in both the male and female pronuclei, and their fusion is not essential to the initiation of this process.

Immediately after mitosis and during interphase the multiplying cell shows a period (G-1) during which the synthesis of DNA does not occur. This is followed by a period of interphase (S) during which DNA synthesis is initiated and proceeds at a constant rate. Subsequently, however, a period (G-2) is encountered during which the synthesis of DNA again is interrupted (61). This phenomenon is general for most protozoan and metazoan cells, as well as for yeast cells (62)—the main differences being the time relationships for the various periods. Nygaard *et al.* (63) have shown that in the slime mold, a multinucleate organism in which cells have lost their individuality and the protoplasm forms a continuum, DNA synthesis starts almost immediately after nuclear division. Such synthesis occurs simultaneously in all the nuclei, lasts for one hour and then stops. The next nuclear division does not occur for 12 to 20 hours. In some cases, a secondary burst of DNA synthesis is also observed [this has been observed with other cells as well, cf. (14)]. Siskin *et al.* (64) report that imposition of a change in generation-time upon a population of cells affects only the time of "telophase plus the interval from the end of telophase to the initiation of DNA synthesis" (T+G-1) and that once the synthesis of DNA commences the cells tend to divide at a constant rate. X-ray irradiation of S-3 HeLa cells (65) and of mouse L-cells (66) indicates that the synthetic period S is unaffected and that a block is produced in the premitotic period G-2. However, the response of cells to x-ray irradiation may also differ with cell type (65). Amand *et al.* (67) find that the rate at which grasshopper embryonic cells pass through prometaphase, early telophase, and middle telophase is accelerated by the addition of agmatine to the culture medium (cf. 68).

In the nucleus of *Euplotes* it appears that a reorganization of DNA occurs at the region of DNA synthesis leading to a dispersion of the DNA. This is assumed to be due to an unfolding of the chains of DNA prior to their duplication (69). A dispersion of nuclear DNA during the life cycle of mature egg cells of ferns and plants has also been noted by Bell (70). He has followed the synthesis of DNA throughout oögenesis in ferns and has found that in the mature egg the DNA appears to become dispersed throughout the cytoplasm of the cell—a circumstance which results in a great dilution of the DNA. Under these conditions the cells appear to be Feulgen-negative. Also, it appears that the rate of cell division of a variety of cells can be enhanced by the addition of small amounts of DNase, as shown by Kiefer *et al.* (71). Relevant to these phenomena may be the experimental findings of Chayen

(72). These are derived from an experimental procedure which permits the disruption of plant cells under rigorously controlled conditions. Using meristematic cells of roots of *Vicia faba*, the DNA can normally be found associated with the cytoplasmic granules and not with the nuclei. Migration of DNA to the nucleus occurs during differentiation and under certain experimental conditions.

In bacterial cells.—With bacteria, the relationship of the synthesis of DNA to the cell cycle does not appear to be clearly defined. Because much of the work has been done with cell cultures that exhibit synchrony in their growth (produced by different experimental means), Abbo & Pardee (73) have differentiated between synchronous cultures and synchronized cultures. They define synchronous cultures as those which divide simultaneously as a result of procedures which do not impose a temporary stress, while they define synchronized cultures as those which divide simultaneously as a result of procedures which remove an existing physiological stress. They consider the two types of cells to be quite different in their metabolic behavior.

In a careful study Abbo & Pardee find that DNA synthesis, in *E. coli* cells selected for synchronous growth, is continuous despite the obvious step-wise increase in cell number. In a culture of *L. acidophilus* which has been synchronized by the withdrawal of thymidine from the medium, Burns (74) suggests that DNA synthesis occurs in a step-wise manner. The stress applied or released may modify the time relationships in the synthesis of DNA in a bacterial culture, as is evidenced by the results of Lark (75). These results appear to indicate that the addition of deoxyribonucleosides to a synchronized culture of *E. coli* can convert the synthesis of DNA from a step-wise phenomenon to a continuous phenomenon. In addition, the author indicates that not only the number of deoxyribonucleosides added, but also the time of addition, is important for eliciting this change. Fitz-James (76) has suggested that the continuous synthesis of DNA observed in bacteria may be related to the fact that each chromatin-containing structure of the bacterial cell duplicates out of phase (cf. synchronous nuclear division in slime molds). The association of the DNA of *E. coli* with two (77) or more (78) large subunits which are distributed among the progeny in a nonrandom fashion has been indicated recently by radioautographic techniques.

The replication of DNA is generally assumed to occur by a semiconservative mechanism. This necessitates that the parental DNA be equally distributed among the DNA of the daughter cells. Working with ϕ X174 and with bacteriophage T₄, Kozinski (79, 80) finds that when these infect *E. coli* cells and multiply, only a small portion of the parental phage DNA is transferred to the DNA of any one of the progeny phage. His results lead him to conclude that the replication of DNA in these cases cannot be semiconservative alone, but also must include a mechanism which allows for the fragmentation of the parental phage DNA and its redistribution to the progeny phage. A physical exchange of sections of DNA, which involves some type of fragmentation process, occurs during the genetic recombination

of phage, as is indicated by the experiments of Meselson & Weigle (81) and those of Kellenberger *et al.* (82). This particular process apparently can occur in the absence of DNA synthesis or the replication of phage DNA.

Two enzymes may be considered to be related to this fragmentation of DNA. One is the type of DNase, which also occurs in calf thymus, obtained from chicken erythrocytes by Bernardi and Sadron and their co-worker, Champagne (83, 84). This degrades DNA into homogeneous large-molecular-weight subunits (described on page 294). The other is an enzyme of calf thymus nuclei which incorporates a limited number of deoxyribonucleotides into DNA. This enzyme may participate in some limited repair reaction at the ends of such fragmented DNA chains (Krakow *et al.* 85).

TIME RELATIONSHIP IN DNA, RNA, AND PROTEIN SYNTHESIS

In protozoan and metazoan cells.—In studying the relationship of DNA synthesis to the synthesis of RNA and protein in the nucleus of the protozoan *Euplotes*, Prescott & Kimball (69) have extended the observations of Gall (86) and of Faure-Fremiet *et al.* (87). Their findings (69) suggest that the synthesis of RNA in the nucleus is depressed or absent in the zone of DNA synthesis, while histidine incorporation is heavy only in the region of DNA synthesis. A depression of RNA synthesis during the time of DNA synthesis in the nuclei of the root tips of *Trandescandia paludosa* has been noted by Sissen (88), although these data indicate that the rate of protein synthesis remains constant in these nuclei at all times.

The results obtained with slime molds (63) suggest that a depression of the synthesis of RNA may occur during the time of DNA synthesis. Woodard *et al.* (14) find high rates of accumulation of chromosomal and nucleolar proteins, as well as of cytoplasmic and nucleolar RNA, in the root tips of *Vicia faba* at those periods when the synthesis of DNA is minimal. At the time of maximal DNA synthesis, the synthesis of histone also is at a maximum, but only a minimum increase of other protein and RNA occurs. A different type of relationship has been described as occurring in *Paramecium aurelia*. In this organism, Woodard *et al.* (89) find that, coincidentally with the increase in DNA synthesis which occurs at mid-interphase, a sudden increase occurs in the rate of synthesis of nuclear protein, cytoplasmic protein, and cytoplasmic RNA. The concurrence of the increased rate of these reactions with the increased rate of DNA synthesis which occurs in this organism may reflect the demands for the duplication and differentiation of several organelles occurring at this time. Under conditions of synchronous growth, a continuous synthesis of the total cellular nucleic acids occurs in the algae, *Chlorella pyrenoidosa* (90).

In bacterial cells.—In synchronous cultures of *E. coli*, Abbo & Pardee (73) find that the synthesis of DNA, RNA, and protein occurs at a linear rate. On the other hand, in synchronized *L. acidophilus*, Burns (74) finds that the synthesis of RNA and protein takes place during the time of DNA synthesis, while an increased rate of synthesis of RNA and protein occurs at the time of

division. During the germination of spores of *B. subtilis*, grown under conditions which permit a fair degree of synchrony, an increased rate of synthesis of RNA, which occurs coincidentally with an increased rate of DNA synthesis, has been observed by Woese & Forro (91). For two hours following germination, RNA synthesis but no DNA synthesis is observed. Subsequently, the initiation of DNA synthesis takes place and, at approximately the same time, there occurs a corresponding enhancement in the rate of synthesis of RNA.

In order to find a common denominator for the foregoing observations on the variety of cell types, it may be postulated that either the synthesis of RNA or that of protein, or both, is required to initiate, but not to sustain the synthesis of DNA. This conclusion is derived from experiments of Maaloe and Hanawalt *et al.* (92, 93), which were carried out with auxotrophs of *E. coli*. These investigations indicate that inhibition of the synthesis of protein and RNA prevents the initiation of a new cycle of DNA synthesis, but that once the latter is initiated it can continue to completion without the concurrent synthesis of protein or RNA. Similar conclusions have been arrived at by Doudney (94).

NUCLEOTIDE POOLS AND THEIR RELATIONSHIP TO NUCLEIC ACID METABOLISM

The ribonucleotide pool.—Franzen & Binkley (95) have measured the acid-soluble nucleotide pool of *E. coli*, grown at different growth rates. They find that the ratio of AMP:ADP:ATP is 1:6:50, regardless of the rate of growth. As the mean-cell size increases, the amount of ATP per cell increases in such a way that the concentration of ATP remains constant. On the other hand, the GTP+UTP content increases at a faster rate, indicating that the larger cells which also grow at a faster rate have a higher concentration of GTP+UTP. Studies on the rate of equilibration of pools in *E. coli* have been made by McCarthy & Britten (96), who demonstrate the existence of two pools of RNA precursors: a small pool which provides precursors for RNA synthesis with little delay and a large but separate pool which is in slow equilibrium with the first. (For further discussion on pool equilibration see page 273.)

Davies, Harris, and Neal (97, 98) have investigated the metabolism of the peptidyl nucleotides of yeast and find that adenine and uracil are rapidly incorporated into these compounds. In addition, the concentration of these complexes increases to a maximum at the onset of growth and declines to a low value during the ensuing period of logarithmic growth. When the rate of growth decreases, however, the amount of peptidyl nucleotides again increases, and subsequently falls when the culture ages. These authors postulate that peptidyl nucleotides are synthesized in excess by the yeast during early growth and then undergo very rapid turnover during logarithmic growth. Thus, at this stage, their stationary concentration at any one time is

low. Subsequently, as growth becomes unbalanced, the synthesis of peptidyl nucleotides continues, but these compounds are now no longer integrated with others in biosynthetic processes, and hence accumulate. Whether these compounds can participate as direct precursors in the biosynthesis of nucleic acids is not known. It is very probable that the component purine and pyrimidine bases of the nucleotidyl peptides are eventually incorporated into nucleic acid. For example, it has been shown that the thymine of thymidine diphosphate-rhamnose is incorporated into DNA (99), but there is no evidence that the polymerization reaction involves this carbohydrate complex as a proximal precursor of DNA.

The deoxyribonucleotide pool.—Attempts have been made to correlate the variations in the size of the acid-soluble deoxynucleoside and deoxynucleotide pools in animal tissues with variations in the rate of DNA synthesis. The following generalizations (100 to 105) can be made: (a) the size of this pool varies from 13 μ g of thymidine-equivalent/g of rat liver to 85 μ g of thymidine-equivalent/g of rat thymus, and is usually larger in actively growing tissues; (b) the deoxyribonucleosides always constitute the larger proportion of this pool, but in the actively growing tissues the deoxyribonucleotide fraction appears to increase; (c) the main components of this pool are pyrimidine compounds, with deoxycytidine accounting for the largest fraction, while its phosphorylated homologues (dCDP-choline, dCDP-ethanolamine) account for a small proportion; (d) the mono-, di-, and triphosphates of thymidine and of deoxycytidine have been identified in thymus extracts; to date, of the purine deoxynucleotides, small amounts of dATP have been identified only in the acid-soluble extracts of the Flexner-Jobling carcinoma; and (e) deoxycytidine and 5-methyl-deoxycytidine account for 80 per cent of the urinary excretion of deoxyribose-containing compounds by the rat, while deoxyuridine accounts for 10 to 15 per cent.

Stern (106, 107) finds that developing lily anthers appear to accumulate deoxyribonucleosides periodically. This accumulation coincides with the time of degradation of DNA, which occurs in this plant from the breakdown of tapetal tissue apparently preceding mitosis and itself preceded by corresponding fluctuations in DNase activity. Stern's results lead him to conclude that the periodic induction of DNase activity is a mechanism for the morphogenetic development of the pollen mother cells. It is still very difficult to evaluate these results fully, because of the complexity of the tissue and the peculiarly intricate interrelationship of the various cell types.

Working with bacteria under conditions which permit the periodic synthesis of DNA and RNA to occur, Lark (108) has studied the variations in the size of the deoxyribonucleotide precursor pool and its relationship to DNA synthesis. These findings may be summarized in the following manner: (a) The acid-soluble deoxyribonucleotide pool appears to be composed largely of phosphorylated derivatives of thymidine and also contains some deoxycytidine. From the work of Kuriki & Okazaki (109) the probable

composition of the thymine pool of growing *E. coli* can be derived: thymidine diphosphate-rhamnose accounts for 45 per cent of this pool, while thymidine and thymidylic acid account for 35 per cent; TDP and TTP account for no more than 10 per cent. (b) The pool size is very small compared to the DNA content. (c) A step-wise increase in pool size occurs which precedes the synthesis of DNA, but the latter does not decrease the size of the pool. (d) The step-wise increase in pool size persists even under conditions which enforce a continuous rate of DNA synthesis.

The absence of deoxyribose derivatives of the purines and the preponderance of the deoxyribonucleosides, rather than the deoxyribonucleotides, in the acid-soluble pool in animal and plant tissues, at first glance appear to speak against the deoxyribonucleosides as the main source of precursors for the synthesis of DNA. On the other hand, Reichard, Canellakis & Canellakis (110) have found that the reductive conversion of CDP→dCDP is inhibited by the deoxyribonucleotides of guanine, adenine, and thymine while the reduction of GDP→dGDP is inhibited by the deoxyribonucleotides of guanine and adenine (the latter reaction is actually enhanced by dCTP and dTTP). This inhibition occurs in the presence of very low levels of the deoxyribonucleotides ($10^{-6}M$) and ultimately results in the inhibition of DNA synthesis. Consequently, the absence of large amounts of these deoxyribonucleotides from the acid-soluble pool is not surprising. It should be noted that in *E. coli* in which the thymine compounds are the main components of the acid-soluble deoxyribonucleotide pool, TDP and TTP account for no more than 10 per cent of this fraction. The characteristics of the inhibition of this reductive reaction would therefore necessitate that the deoxyribonucleoside diphosphates formed from the corresponding ribonucleoside diphosphates be either immediately and efficiently used in the synthesis of DNA or be stored in a noninhibitory form, i.e., as deoxyribonucleosides. In this case, the availability of an efficient mechanism for phosphorylation of the deoxyribonucleosides and of a mechanism for their rapid utilization becomes most critical. Such a concept is compatible with the idea that thymidine kinase (111) and thymidylate kinase (112, 113) could play a key role in the initiation of DNA synthesis in regenerating rat liver. Consequently, it appears attractive to postulate that the rate of synthesis of the deoxyribonucleotides from their ribonucleotide precursors is regulated by the concentration of the deoxyribonucleotides. This could provide a rate-limiting step in DNA synthesis. It has been suggested by Morris & Fischer (114) that the inhibition of the conversion of ribonucleotides to the deoxyribonucleotides, mentioned earlier, with the consequent inhibition of DNA synthesis, accounts for the inhibition of growth of cell cultures by thymidine. Overgaard-Hansen & Klenow (115) find that the inhibition of the synthesis of DNA by deoxyadenosine may be attributable to such an effect; similar conclusions are arrived at by Munch-Petersen (116). In chick embryos, the inhibition by dAMP of the formation of DNA from ribonucleotide precursors, observed by Maley & Maley (117), also may be explained on this basis.

REGULATION OF NUCLEIC ACID SYNTHESIS

The variability of the activity of enzymes associated with the biosynthesis of precursors of DNA has been examined in animal tissues under various conditions of growth. It has been found that, whereas the activity of the kinases for dCMP, dAMP and dGMP varies only slightly under various conditions of growth (113), the activity of thymidine and thymidylic acid kinases increases from very low values in normal rat liver to high values in regenerating rat liver (110, 111, 116). An attempt also was made to establish whether this variation in enzyme activity may be the result of enzyme induction by the substrate. This was done by measuring the level of these enzymes in the normal rat liver subsequent to the administration of thymidine to rats. Later, a large proportion of the observed increase in enzyme activity was found by Bojarski & Hiatt (117) to reflect a stabilization of the enzyme by the substrate. Weissman *et al.* (118) find that in regenerating rat liver there occurs a sequential appearance of the kinases associated with the step-wise phosphorylation of thymidine to thymidylic acid to thymidine diphosphate to thymidine triphosphate. A similar phenomenon was observed in cultures of L-strain fibroblasts and Weissman and his co-workers suggest that this may be compatible with enzyme induction. Bianchi *et al.* (119) have studied the rate of phosphorylation of thymidine to thymidine triphosphate and suggest that thymidine is phosphorylated to thymidylic acid, that this is then pyrophosphorylated to thymidine triphosphate, and that thymidine diphosphate is formed from the latter. Gray *et al.* (120) show that extracts of normal tissues contain nondialyzable factors which act as inhibitors to the reactions described above and provide evidence that the inhibitory action is not the result of dephosphorylation by phosphatases. Because of this latter finding, the evaluation of the details of the enzymatic reactions described above, as well as of the mechanism underlying the variation in enzyme activity, should await more extensive enzyme purification.

Scarano *et al.* (121) and the Maleys (122) have found that deoxycytidylic acid deaminase, an enzyme which provides dUMP as a precursor for the synthesis of TMP, varies from low but measurable amounts in normal tissues to high levels in the regenerating rat liver of other tissues undergoing active growth. The existence of this enzyme in normal liver and in the Dunning hepatoma has been questioned (123). However, this problem appears to have been resolved by the use of more sensitive techniques which demonstrate the presence of the enzyme in these tissues (124, 125). Similar variations in the levels of dCMP deaminase with the state of growth have been demonstrated by Myers *et al.* (126). A microsomal inhibitor of dCMP deaminase, which appears to inhibit dCMP deaminase specifically (it does not inhibit deoxyadenosine deaminase), has been described by Fiala & Fiala (127). Its ease of extraction differs from tissue to tissue: extracts of an acetone powder prepared from Ehrlich ascites cells contain low amounts of the inhibitor, while similar extracts of acetone powders of the Novikoff hepatoma contain appre-

cialable amounts of the inhibitor. Stabilization of dCMP deaminase against the action of this inhibitor occurs in the presence of dCMP. The existence of such an inhibitor of dCMP deaminase may account for the variability in the results obtained from various laboratories with respect to this enzymatic activity, and emphasizes the difficulties inherent in the comparison of enzymatic activities in different tissues. The similarities between the enzymes described above, which have been emphasized in this discussion for comparative purposes, suggest that perhaps the levels of enzyme remain relatively constant and their differential activity is the resultant of stabilization by substrates and the variability of levels of inhibitors.

Another enzyme, the function of which may be associated with the synthesis of thymidylic acid, and whose activity is found to vary with the state of growth of the tissue, is 5-methyl-uridylic synthetase, which methylates UMP in the presence of formaldehyde to the ribonucleotide analogue of thymidylic acid (128) (see page 289). The activity of thymidylic acid synthetase, the enzyme which converts dUMP to thymidylic acid, has been shown by Maley & Maley (129) to be increased in growing tissues and in general to follow variations of activity similar to those of dCMP deaminase. These two enzymes form one section of the link in the transformations which occur between cytidylic acid and thymidylic acid. Since Reichard *et al.* (130) have shown that the reduction of cytidylic acid to dCMP occurs at the diphosphate level, the whole sequence of associated reactions may be written as follows: $\text{CMP} \rightarrow \text{CDP} \rightarrow \text{dCDP} \rightarrow \text{dCMP} \rightarrow \text{dUMP} \rightarrow \text{TMP}$: an alternative sequence being $\text{UMP} \rightarrow \text{UDP} \rightarrow \text{dUDP} \rightarrow \text{dUMP} \rightarrow \text{TMP}$. Of the enzymes involved in these sequences, the recent work of Sköld (131) emphasizes the elevated activities of the kinases in rapidly growing tissues. In addition, although no comparative assay of the activity of the enzyme associated with the reductive step has been made, it can be demonstrated most easily in extracts of actively growing tissues.

A corollary to the increased anabolic activity of growing tissues is their decreased ability to degrade precursors of nucleic acids to compounds which cannot be used directly for these anabolic purposes. Stevens & Stocken (132) find that fetal rat liver does not appear to be capable of degrading uracil and thymine. With increasing age of the animal, the activity of the degradative pathway increases, reaching its maximum in the livers of adult animals. Related observations have also been published (133). In the context of the preceding discussions, it should be mentioned that the catabolism of pyrimidines is also inhibited by a microsomal factor (134).

Wheeler & Alexander (135, 136) find that tumors have a decreased capacity to degrade purines and an increased ability to anabolize these compounds, as compared to these capacities of normal host tissues. Their results lead them to the conclusion that xanthine oxidase is limiting in these abnormal cells (cf. 137).

To date, there is no direct evidence that within the normal cell cycle the rate of nucleic acid synthesis is controlled by alterations of the size of the

precursor pools (see page 282), or by alterations in the amount or the activity of enzymes associated with the various steps leading to the synthesis of nucleic acids. Evidence exists that cells growing at different rates often demonstrate alterations of the size of pools of precursors and of enzymatic activities; these changes are in accord with the increased demands imposed upon the cell for nucleic acid synthesis.

The complexity of the problem is further emphasized by the finding of Allfrey *et al.* (138) that the incorporation of adenine, adenosine, and uridine into the RNA and of thymidine into the DNA of calf thymus nuclei, as well as the transport of these compounds into these nuclei, is dependent upon the concentration of Na^+ . Relevant articles have been published recently on cellular regulatory mechanisms (139) and on the alteration of the host-enzyme pattern after infection by bacteriophages (140).

NUCLEOTIDE METABOLISM

As mentioned previously, it has been established by Reichard *et al.* (130) that the reduction of ribonucleotides to the corresponding deoxyribonucleotides occurs at the nucleoside diphosphate level. In addition Bertani, Häggmark & Reichard (141) have shown the dependence of the reaction on ATP, triphosphopyridine nucleotide (reduced form), and on high levels of lipoic acid in the presence of partially purified enzyme preparations from *E. coli*. These extracts also can convert UDP to dUDP which can be further phosphorylated to dUTP and contain a specific pyrophosphatase which very rapidly cleaves the dUTP; it is attractive to regard this as a "protective" enzyme which minimizes the possibility of introducing dUMP into DNA.

The studies of Hall & Allen (142) on the biosynthesis of 5-ribosyluracil indicate that cytidine is an effective precursor of this compound in a pyrimidine-requiring strain of *Neurospora crassa*. Although the exact sequence of reactions is not known, evidence is presented that uracil is not an intermediate. Lis & Allen (143) report that the enzymatic amination of 5-ribosyluracil leads to the synthesis of 5-ribosylisocytosine. The properties and mode of action of orotidylate decarboxylase have been studied by Creasey & Handschumacher (144) and by Blair & Potter (145). Blair & Potter have studied the inhibition of the orotidylate decarboxylase of rat liver extracts and find that it is competitively inhibited by UMP, while uridine and UTP have no effect on enzyme activity; the effect of UDP is debatable. Creasey & Handschumacher (144) have partially purified the enzyme from rat liver and from brewer's yeast and have made a comparative study of the two enzymes. They find that the purified rat liver enzyme is competitively inhibited by UMP and much less so by CMP; uridine, UDP, and UTP are inactive. The brewer's yeast enzyme is competitively inhibited by UMP, CMP, AMP, and GMP (in decreasing order of inhibition); the two enzymes are very different with respect to their requirement for sulfhydryl groups and their sensitivity to alkaline and acid levels of pH.

Using rats, Batzer & Schweigert (146) administered CMP labeled in both

the ribose and the base and found that the UMP isolated from the RNA had exchanged a large portion of the ribose. They suggest that some mechanism for the conversion of CMP to UMP other than through direct deamination of cytidine may exist. Alternative explanations involving differences in pool sizes may also explain this phenomenon. Maley & Maley (147) have shown that minces of chick embryo can convert uniformly labeled dAMP to dGMP without a change in the ratio of the label in the base to that of the label in sugar, indicating that the conversion can occur without prior depurination.

Cabib & Carminatti (148) have identified a nucleotide pyrophosphatase in yeast which hydrolyzes the pyrophosphate linkage of GDP-mannose, UDP-glucose, UDP-acetylglucosamine, and of diphosphopyridine nucleotide. Studies on the distribution of enzymes associated with nucleotide metabolism indicate that the mitotic apparatus contains an ATPase which is specific for ATP (it has no effect on GTP, UTP, or CTP) (149), and that the Golgi apparatus contains a nucleoside diphosphatase which acts preferentially on IDP, GDP, and UDP and exhibits a lower activity towards CDP and IDP (150).

Chiga & Plaut (151), using swine liver as a source, have purified extensively an ATP-AMP transphosphorylase which is specific for the adenine nucleotides. Chiga, Rogers & Plaut (152) also have purified from swine liver a GTP (or ITP)-AMP transphosphorylase which catalyzes only the reaction between the oxypurine nucleoside triphosphate and adenylic acid. Both these enzymes will catalyze their corresponding transphosphorylase reactions at a slower rate if one of the reaction components is replaced with the corresponding deoxyribonucleotide. In neither case does the reaction proceed if both components have been replaced with the corresponding deoxyribonucleotides. The authors believe that they are isolating the individual enzymes involved in the reaction: $XTP + AMP \rightleftharpoons XDP + ADP$. This reaction has been demonstrated previously in calf liver (153, 154). A variety of nucleoside mono- and di-phosphokinases have been partially purified from *Ascaris lumbricoides* and the specificity is shown to reside in the base and not in the sugar (155).

The dGMP kinase of *E. coli*, which is activated by K^+ , has been purified by Bello, Van Bieber & Bessman (156). This enzyme is chromatographically distinct from the dGMP kinase of *E. coli* infected with either T2, T4, or T6 bacteriophage, which is inhibited by K^+ . Zimmerman & Kornberg (157) have shown that such bacteriophage-infected *E. coli* also have an enzyme which is specific for the degradation of dCTP and dCDP. dCTP is cleaved to dCMP and pyrophosphate, while the same enzyme cleaves dCDP to dCMP and phosphate (cf. 158). Kornberg *et al.* (159) have shown that these bacteriophage-infected *E. coli* also contain hydroxymethylcytosine- α -glucosyl transferases, enzymes which transfer glucose from UDPG to the 5-hydroxymethylcytosine residues in the DNA in α -linkage. The enzyme present in *E. coli* cells infected with T₄ bacteriophage can be distinguished from the

corresponding enzymes present in cells infected with T4 and T6 bacteriophage. The T4-infected cells produce, in addition, a hydroxymethylcytosine- β -glucosyl transferase, an enzyme which forms similar monoglucosyl units in β -linkage, whereas the T6-infected cells produce, in addition, an enzyme which adds a second glucosyl unit in β -linkage to a pre-existing monoglucosylated 5-hydroxymethylcytosine residue.

From calf thymus, Greenberg *et al.* (160) have purified thymidylate synthetase, an enzyme which methylates dUMP in the presence of formaldehyde and tetrahydrofolic acid. They provide evidence which implies that thymus extracts contain an isomerase which converts the tetrahydrofolic acid oxidation product to 7,8-dihydrofolic acid, the form that is presumed to be the substrate for 7,8-dihydrofolic acid reductase. Such an isomerase does not appear to be necessary for the similar reaction catalyzed by extracts of *Streptococcus faecalis*, studied by McDougall, Blakley, and Whittaker (161, 162) (cf. 163). The difference in enzymatic capacities for the synthesis of thymidylic acid present in extracts of prototrophs and thymine auxotrophs of *E. coli* has been investigated by Mantsavinos & Zamenhof (164). Their experiments also provide evidence for the enzymatic synthesis of thymine riboside from thymine by these extracts.

The pathways in *E. coli* which result in the biosynthesis of thymidine in DNA and of thymine riboside in S-RNA appear to differ significantly. While the methyl of thymidine is provided through formaldehyde and tetrahydrofolic acid, the methyl of thymine riboside appears to be derived from methionine (165). This implies that interconversion of these two forms of thymine, whether by exchange or alteration of the sugar moiety, is improbable. Consequently, it appears that the thymine riboside which is synthesized in the presence of UMP and formaldehyde (128) is more likely to be related to thymidine in DNA than to thymine riboside in S-RNA (cf. page 286). The methyl group of methionine appears also to be the precursor of the methyl groups of methyladenine (166) and methylguanine (167). In *Neurospora crassa*, thymidine can be converted in significant amounts of deoxycytidine in DNA and to uridine and cytidine in RNA (168).

NUCLEOTIDE DERIVATIVES

Nucleotides bound to a variety of sugars have been identified in cell extracts. Okazaki (99) has identified TDP-rhamnose in extracts of *L. acidophilus* and *E. coli* and its biosynthesis from TDP-glucose has been studied by Pazur & Shuey (169a) and by Kornfeld & Glaser (169b, 169c). Baddiley *et al.* (170) have synthesized TDP-mannose and TDP-glucose and shown them to be interconvertible by extracts of *S. griseus*. TDP-mannose has also been identified as naturally existing in extracts of *S. griseus*. Su & Hassid (171) have identified guanosine diphospho-L-galactose and its D-mannose analogue in extracts of the red algae, *Porphyra perforata*, as well as adenosine 3',5'-pyrophosphate. Ginsburg (172) has isolated guanosine-

diphospho-D-glycero-D-mannoheptose or its enantiomorph from yeast. Sukuzi (173) has isolated UDP-glucose-N-acetyl-P-galactose from hen oviduct. The isolation from pig's blood of AMP linked to 2,3-diphosphoglyceric acid by a pyrophosphate bond has been described by Nashimoto *et al.* (174). Khalidi & Greenberg (175) have identified guanine propionate [2-(α -propionamino)-6-hydroxypurine] from acid hydrolysates of extracts of *Eremothecium ashbyii*; their experiments lead them to suggest that it may be an intermediate in riboflavin biosynthesis.

Recently a number of peptidyl-nucleotides have been identified in which the amino acid is linked to the nucleotides by an anhydride link between the carboxyl group and the phosphate group. Davies & Harris (176, 177, 178) have identified a large number of such compounds in yeast extracts and show them to consist of a great variety of types, i.e., simple nucleotides linked to a single amino acid or to polypeptide chains of variable lengths. In addition, di- or tri-nucleotides linked to single amino acids or to peptides have been isolated. Adenylic acid, uridylic acid, and cytidylic acid have been identified as components of such compounds. Harris & MacWilliams (179) have published a method for the synthesis of some of these compounds. Schuurs and Koningsberger (179) find such cytidylic acid peptides in yeast extracts and have also provided evidence (180) for the enzymatic synthesis of activated peptides although the exact structure of these compounds has not been established. Similar compounds have also been found in rat liver extracts (182). Szafranski & Bagdasarian (183) have found that the amino acids of peptidyl-nucleotides, when incubated with rat liver microsomes and the pH 5 fraction, are incorporated into the protein of the soluble proteins, but not into that of the microsomal protein. A peptide linked to adenosine-3',5'-diphosphate in a nonanhydride link has been isolated from bovine liver by Wilken & Hansen (184). Comb *et al.* (185) describe the isolation from *E. coli* of a uridine nucleotide-complex of α , ϵ -diaminopimelic acid. Nucleotide peptides have been identified in RNA (186). Medvedev *et al.* (187) find that plant extracts contain polynucleotides as well as polypeptides which demonstrate a very high rate of turnover.

INTERFERENCE WITH NUCLEIC ACID METABOLISM

Reference will be made to those aspects of this problem which can be closely related to the topics previously discussed.

Ultraviolet-irradiation.—Beukers & Berends (188) have shown that ultraviolet irradiation of rapidly frozen solutions of thymine leads to the formation of dimers of thymine; if the ultraviolet irradiation is repeated on this solution after it has been thawed, the reaction is reversed and thymine can be recovered. These researchers postulate that rapid freezing brings the molecules of thymine sufficiently close together that, upon subsequent irradiation, dimerization is possible. A formation of such dimers also has been accomplished by Wacker *et al.* (189). Following the irradiation of

Enterococci, and the degradation of the bacterial DNA, these investigators have isolated thymine dimers. Under their conditions of irradiation, as much as 16 per cent of the total thymine can be converted to the dimeric form. Interestingly enough, the structure they present for the thymine dimer (covalent bonds linking carbons 5 to 5 and 6 to 6) is a *cis*-isomer of the *trans* structure presented by Beukers & Berends (covalent bonds linking carbons 5 to 6 and 6 to 5). If this difference can be substantiated, it may be attributable to the difference in orientation of thymine in solution, as compared to that of the thymine in the chains of DNA. Using as an assay system the reactivation of ultraviolet-inactivated *Hemophilus influenza* transforming DNA, Rupert (190) has been able to isolate from bakers' yeast a fraction which causes 50 per cent photoreactivation of the transforming properties of this ultraviolet-inactivated DNA. This factor has been shown to be a photo-reactivating enzyme (PR) which catalyzes a light-dependent reaction. Marmur & Grossman (191) have studied the action of this purified PR enzyme on ultraviolet-irradiated DNA and suggest that 50 per cent of the action of ultraviolet light on DNA may be attributable to the formation of the thymine dimers described above. This effect is reversed in the presence of the PR enzyme. Their results indicate that treatment of double-stranded, ultraviolet-irradiated DNA with the PR enzyme permits its conversion to a single-stranded DNA, and suggest that the cross-linking of DNA strands produced by ultraviolet irradiation may occur through the formation of such dimers. The development of cross-linking between strands of DNA *in vivo* may render the DNA biologically inactive, because of the inability of the strands to separate during replication or prior to exerting a template role in the synthesis of RNA.

The results of Hanawalt & Setlow (192) also emphasize the effect of ultraviolet light on DNA. These results show that, at the low doses of irradiation which inhibit the synthesis of DNA by *E. coli*, there is little or no inhibition of protein synthesis. At higher doses of ultraviolet irradiation, however, the synthesis of protein appears to be more sensitive than does that of RNA. Drakulic *et al.* (193) find that if chloroamphenicol is added to *E. coli* immediately after irradiation, in order to inhibit protein synthesis, large losses of intracellular nucleic acids occur, as well as a permanent inhibition of the synthesis of protein. In contrast, if the addition of chloroamphenicol is delayed, the losses of intracellular nucleic acids do not occur and DNA synthesis resumes. These results and others are in apparent agreement with the experiments of Hanawalt and Maaloe and their co-workers (92, 93) which indicate that initiation of the synthesis of DNA is dependent upon the synthesis of RNA and protein. Doudney (194) has extended these observations and suggests that the synthesis of RNA and protein, which is necessary for the initiation of DNA synthesis after ultraviolet irradiation, permits the transfer of information from the double stranded, ultraviolet-inactivated DNA to an intermediate RNA-protein structure, and that this functions as a

template in the subsequent formation of DNA. It should be noted that Suzuki & Ono (195) find that, although ultraviolet irradiation may not inhibit the rate of the synthesis of RNA, the RNA synthesized after irradiation is metabolically unstable and participates in extensive turnover (cf. 196).

Some further insight into the mode of action of ultraviolet irradiation and on the mechanisms of nucleic acid synthesis may be derived from the ultraviolet irradiation of cells which have been grown in the presence of various analogues of nucleic acid precursors. Greer (197) and Kaplan *et al.* (198) find that the following compounds, when incorporated into the DNA of *E. coli*, enhance the sensitivity of the cells to ultraviolet irradiation (in decreasing order of effectiveness): thioguanine, 5-bromo-2'-deoxyuridine, 5-bromouracil, 5-iodouracil, and 2,6-diaminopurine. These findings appear to be related to the properties of the compounds themselves, rather than to the amount incorporated into nucleic acids, because thioguanine, which is the most effective analogue, is incorporated into DNA to only a small extent. Again extensive replacement of the thymine of DNA with 6-methyl-aminopurine, as happens in *E. coli* 15T⁻ grown under conditions of thymine starvation, does not alter the sensitivity of the cells to ultraviolet light. On the other hand, the effectiveness of 5-bromouracil in enhancing the sensitivity to ultraviolet irradiation is proportional, within limits, to the amount of 5-bromouracil incorporated into the DNA. The great effectiveness of thioguanine makes it probable that the mechanisms for this phenomenon may be more complex. When 5-bromouracil-containing bacterial cells are irradiated with ultraviolet light, they show less response to photoreactivation than do normal cells, while no photoreactivation of 5-bromouracil-containing T2 bacteriophage can be demonstrated subsequent to ultraviolet irradiation (199). Analogues, e.g., 2-thiothymine, 5-fluorouracil, and 2-aminopurine, which are not incorporated into DNA, do not affect the ultraviolet sensitivity of bacterial cells. Of these compounds, 5-fluorouracil is extensively incorporated into RNA—a finding which strongly implies that such modification of RNA does not lead to increased sensitivity to ultraviolet irradiation [cf. effect of ultraviolet light on 5-ribosyluracil (200)].

There appears to be a difference between mammalian cells and bacterial cells in their response to ultraviolet irradiation. Djordjevic & Szybalski (201) find that mammalian cells grown in the presence of 5-fluoro-2'-deoxyuridine and 5-bromo-2'-deoxyuridine, and thus heavily labeled with 5-bromouracil in place of DNA-thymine, exhibit much greater sensitivity to ultraviolet irradiation than do normal cells, and conclude that labeling of both strands of the DNA is necessary for this effect. In contrast, Kaplan *et al.* (202) find that labeling of only one strand of the double helix of *E. coli* is sufficient to confer half-maximal radiosensitization.

Mitomycin C.—Mitomycin C is a relatively new antibiotic agent which will probably be of great help in elucidating the interrelationships of nucleic acid metabolism. Exposure of cells to this compound, which causes the depolymerization of DNA (203 to 212), can result in the loss of as much as

63 per cent of the total DNA within two hours (205), yet, during this time, lysis of cells does not occur and the synthesis of RNA and protein continues. This depolymerization of DNA, which is associated with a release of polydeoxyribonucleotides, is presumed to result from the degradation of DNA to small molecular weight polymers. The inhibition imposed by mitomycin C cannot be overcome by thymine, thymidine, or a mixture of the deoxyribonucleosides of DNA. It does not appear to inhibit the DNA polymerase reaction, since attempts to inhibit the enzymatic synthesis of DNA in extracts of *E. coli* with mitomycin C have been unsuccessful. The action of mitomycin C requires the presence of Mg^{++} and is inhibited by streptomycin. The apparent specificity of action of mitomycin C for the depolymerization of host DNA has suggested its use in testing the extent of participation of host DNA in the synthesis of viral RNA and DNA. It does not inhibit the reproduction of T2, T3, and T5 bacteriophage in *E. coli* or of pseudorabies virus in rabbit kidney cells, although at high concentrations it does inhibit their infectivity. Reich & Franklin (208) find that L-cells in culture will support the growth of Mengovirus (a RNA virus) after a treatment with mitomycin C which inhibits the capacity of the host cells to synthesize DNA and RNA, and destroys the integrity of the DNA of the cells. Accordingly, they conclude that neither the integrity of the host DNA nor concurrent synthesis of DNA is required for the synthesis of viral RNA. This is in agreement with the results of Simon (209), who finds that synthesis of viral RNA occurs in cells in which the synthesis of DNA has been inhibited with 5-fluorouracil and amethopterin. An experiment similar to the one performed with Mengovirus also was performed with vaccinia, a DNA virus; the results of this experiment indicate that vaccinia cannot multiply in cells which have been pretreated with mitomycin C. This finding led to the conclusion that the reproduction of vaccinia is dependent upon an intact DNA of the host cell. These results are not in agreement with those of Cairns (211), who has shown that vaccinia can reproduce in the host cytoplasm and that this reproduction is independent of the host DNA. The inhibition of the reproduction of vaccinia by mitomycin C contrasts with the lack of effect of this agent on the reproduction of the T2 bacteriophages and pseudorabies virus. These differences cannot be evaluated until more knowledge is acquired concerning the mechanism of action of mitomycin C.

NUCLEASES

A study of the distribution of RNases indicates their association with all subcellular fractions of guinea pig pancreas (213). In HeLa cells and in other mammalian cells maintained in culture, RNase activity has been demonstrated in the cell membrane (214). In pea seedlings, Matsushita & Ibuki (215) find RNase activities associated with both the endoplasmic reticulum and the ribonucleoprotein particles. High RNase activities of suspensions of these particles can be demonstrated without prior treatment with ethylenediaminetetraacetic acid or urea, as is required for the demonstration of such

activity in the ribosomes of *E. coli* (216). Spahr & Hollingworth (217) find that all the RNase activity of crude extracts of *E. coli* can be accounted for by the ribosomes (cell walls and membranes are not included in this measurement). The RNase activity is present in the 30S ribosomes of *E. coli*; a content of 0.1 to 0.01 molecule per ribosome has been calculated. This enzyme has been purified extensively and has been shown to hydrolyze all internucleotide linkages to RNA to nucleoside 2',3'-cyclic phosphates which are subsequently cleaved to the 3'-phosphates. In its mode of action, the ribosomal RNase is most similar to that of rye grass; it degrades soluble RNA completely, leaving no unhydrolyzed core RNA. This *E. coli* RNase appears to be a basic protein; it has a pH optimum at 8.1 and is relatively stable to heat treatment at acid pH. In the culture medium of *Bacillus subtilis* (218, 219), relatively large amounts of a variety of RNases are accumulated. Two of these enzymes have been crystallized. They have a pH optimum of 7.5 and hydrolyze only the secondary phosphate esters of the purine ribonucleoside 3'-phosphates in RNA; cyclic GMP and AMP can be isolated as intermediary products. The oligonucleotides isolated after "core" RNA was treated with these enzymes were shown to end in guanylic acid and adenylic acid (cf. 220). The intracellular RNases of *B. subtilis* are of the acid type; very little alkaline RNase can be detected unless the cell lysate is treated with urea. Fellig & Wiley (221) have purified an RNase from *Euglena gracilis* which degrades "core" RNA and which is equally active toward poly U and poly A. Using extracts of *Azotobacter agilis*, Stevens & Hilmo (222) have purified a different type of nuclease. This degrades both RNA and DNA to oligonucleotides with 5'-phosphomonoester end-groups (cf. micrococcal nuclease). This enzyme rapidly degrades poly A to oligonucleotides, but the oligonucleotides are decomposed more slowly, i.e., a pentanucleotide of adenylic acid was degraded to a trinucleotide and a dinucleotide, while a tetranucleotide was degraded more slowly to two dinucleotides. The enzyme degrades poly U and poly C slowly and has no action on poly G.

Williams, Sung & Laskowski (223) have described an improved method for the preparation of snake venom phosphodiesterase. The enzyme catalyzes the degradation of native DNA to mononucleoside 5'-phosphates and exerts a typical exonucleolytic action without exhibiting endonucleolytic activity. A phosphodiesterase with a different specificity has been purified by Lehman (224) from *E. coli*. This enzyme degrades only heated DNA or single-stranded DNA to a dinucleotide and the constituent 5'-mononucleotides, but it does not degrade dinucleotides. It is not inhibited by the presence of glucosylated 5-hydroxymethyl cytosine groups in DNA, and, accordingly, it will degrade heated DNA from T-even bacteriophages. It appears to be an exonuclease with little if any endonuclease activity. Varenko & Laskowski (225) have studied the mode of action of pancreatic DNase on oligonucleotides. The work of Bernardi, Sadron, and Champagne (83, 84) indicates the presence of a DNase in chicken erythrocytes and in calf thymus which degrades high molecular weight DNA (8.0×10^6 to 1.1×10^6) into double

helical rod-like subunits with a molecular weight of 5 to 6×10^5 . The reaction suggests a nonrandom breakdown of the DNA and does not lead to the liberation of detectable amounts of mononucleotides. These workers postulate the existence of a number of discrete sites in the DNA molecule, which connect subunits of similar lengths, and suggest that these sites are susceptible to degradative action by these enzymes according to single-strand degradation kinetics. The highly purified enzyme of chicken erythrocytes also demonstrates a slower, classical DNase-like activity in that it attacks these subunits according to double-strand degradation kinetics; during this step mononucleotides are also liberated.

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ENZYMATIC SYNTHESIS OF NUCLEIC ACIDS^{1,2}

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The author has not attempted to review the entire literature of the past year related to nucleic acid biosynthesis, but instead will discuss rather extensively the enzyme systems associated with polynucleotide synthesis. This selection reflects personal interest and also avoids overlap with the review *Metabolism of Nucleic Acids* in this volume (1). Additional discussion of nucleic acid biosynthesis can be found in several recent reviews (2, 3) (see also reviews in vol. III of *The Nucleic Acids*, Academic Press Inc., New York, 1960).

The last few years have produced notable advances in our understanding of the pathways of nucleic acid biosynthesis. Although an enzyme for ribopolynucleotide synthesis was discovered first, knowledge of the mechanism of RNA biosynthesis is much less complete than it is for DNA. DNA contained within the chromosomes can be regarded as a single biochemical and molecular unit, whereas cellular RNA is physically and metabolically heterogeneous. It may well be, therefore, that in contrast to DNA synthesis, which appears to proceed by a unique mechanism, several different enzyme systems are involved in RNA synthesis.

RIBOPOLYNUCLEOTIDE SYNTHESIS

STRUCTURE AND PROPERTIES OF THE DIFFERENT RNA's

Ribosomal RNA.—Eighty to ninety per cent of the cell's RNA is present in ribonucleoprotein particles (microsomes and ribosomes). The isolation and structure of ribosomal particles from mammalian, plant, and microbial sources has been discussed in a number of recent publications (4 to 11).

The integrity of *Escherichia coli* ribosomal RNA has been re-examined recently by Möller & Boedtker (12) in the light of reports that ribosomal

¹ Most of the literature cited in this review appeared before December 31, 1961, although several papers were in press at the time of writing and appeared subsequent to that date.

² The following abbreviations will be used: AMP, CMP, GMP, UMP, (the monophosphates of adenosine, cytidine, guanosine and uridine); ADP, CDP, GDP, UDP; ATP, CTP, GTP, UTP (the diphosphates and triphosphates of the same); IDP (inosine diphosphate) UMPS (uridine-5'-phosphosulfate); AMPS (adenosine-5'-phosphosulfate) DNase (deoxyribonuclease) RNase (ribonuclease) S-RNA (amino acid acceptor RNA); poly A, poly G, poly U, poly C, poly I, poly BrU, poly CIU, poly IU (homopolymers of adenylic, guanylic, uridylic, cytidylic, inosinic, bromouridylic, chlorouridylic, iodouridylic acids); poly AGUC (copolymer of adenylic, guanylic, uridylic and cytidylic acids) dT, dG, dC (homopolymers of deoxythymidylic deoxyguanylic, deoxycytidylic acids); dAT (copolymer of deoxythymidylic and deoxyadenylic acids).

RNA dissociates into low molecular weight sub-units without apparent breakage of primary chemical bonds. However, no evidence for low molecular weight sub-units was observed. The 16S component contained in the 30S ribosomes represents the minimum size component of ribosomal RNA. The 23S contained in 50S ribosomes would represent a dimer of the 16S. Preliminary results indicate that a third component, 28S RNA, can be isolated. The 28S RNA is particularly labile in the absence of Mg and this might explain the dissociation of 70S ribosomes to 50 and 30S ribosomes in the absence of Mg.

Evidence has accumulated for the participation of ribonucleoprotein particles in protein synthesis and has been extensively reviewed elsewhere (11, 13, 14). One might therefore expect that the genetic information coded in the base sequence of DNA would be transcribed into ribosomal RNA.

Comparison of the base composition of the total RNA among micro-organisms of widely different DNA compositions reveals a surprisingly uniform picture amongst rather unrelated organisms (15). Nucleotide analyses of ribosomal RNA from a variety of sources containing DNA's with different guanine-cytosine base compositions give similar values (13, 16 to 21). Furthermore, the nucleotide composition of RNA is roughly the same in the three different kinds of ribonucleoprotein particles of *E. coli*—30S, 50S, and 70S (17).

All of the other properties of ribosomal RNA, including its homogeneity (see above) and stability (1), argue against its identification as the carrier of genetic information.

Amino acid acceptor RNA.—The existence of a ribonucleic acid fraction (about 10 per cent of the total cellular RNA) in the soluble or nonsedimentable fraction (100,000 g for 120 min) with the unique property of binding amino acids is now well established (11, 13, 22). Its structure and methods of purification have been discussed in detail in a former review (11). Amino acid acceptor RNA (also called transfer RNA or S-RNA) appears to consist of some twenty separate RNA chains, each specific for a different amino acid. The amino acid is linked through its carboxyl group to a ribose hydroxyl group of the terminal adenylic acid residue of its specific RNA chain. Each amino acid acceptor RNA is terminated at the acceptor end by the identical trinucleotide sequence . . . pCpCpA and at the other end by guanosine-5'-monophosphate (23, 24). Studies by Berg & Lagerkvist (25) have demonstrated that the structural similarities amongst the acceptor RNA chains go no further than the pCpCpA trinucleotide end group: For example, the nucleotide sequence adjacent to the pCpCpA end of the RNA chains specific for isoleucine is different from the corresponding sequence in the RNA chains which accept leucine. Preliminary results also indicate a heterogeneity of nucleotide sequences amongst chains specific for a single amino acid. The degradation of only a fraction of the chains specific for a particular amino acid by polynucleotide phosphorylase also supports the existence of such a structural heterogeneity (26, 27).

Estimates of the molecular weight of the amino acid acceptor RNA derived from ultracentrifugation studies (28 to 33) and end group determinations give a chain length between 70 to 90 nucleotides (19, 34, 35, 36). Preliminary results suggest that the molecular weights of the various RNA's specific for different amino acids are the same (30, 31, 32, 35, 37).

The presence of a secondary structure in the amino acid acceptor RNA has been established by studies of the effect of heating on the optical density and optical rotation (28, 33, 38, 39) and from the x-ray diffraction studies (28). Mg^{++} seems to have a protective effect on the secondary structure of S-RNA (39, 40). One of the features of amino acid acceptor RNA (from yeast, rat liver, or *E. coli*) is its resistance to phosphorolysis by polynucleotide phosphorylase (26, 27, 38, 39, 41, 42). The resistant chains (70 per cent of all the chains of S-RNA) have not lost their ability to accept most amino acids (26, 27), which indicates that the chains resistant to polynucleotide phosphorylase have not lost even one nucleotide. The ability of heat (26) and urea (39) to modify partially the extent of phosphorolysis of this S-RNA suggests that this resistance is at least partly due to the secondary structure.

Analyses of the base composition of amino acid acceptor RNA's from a variety of sources show them to differ markedly from RNA's of the ribonucleoprotein particles. One of the features which distinguishes them from other types of ribonucleic acids is the presence in relative excess of a number of unusual purine and pyrimidine bases (16, 18, 34, 43 to 47). Another characteristic of this RNA is a near-equivalence in the molar ratios of adenylic to uridylic and guanylic to cytidylic acids (16, 18, 19, 23, 24, 35, 36, 37, 47, 48). The question is not settled as to whether this equivalence is true for each amino acid specific RNA chain. It appears to hold true in the tyrosine transfer RNA from yeast and *E. coli* isolated by Brown *et al.* on a brushite column (48). However, the correspondence between adenylic and uridylic acid is lost in some of the amino acid acceptor fractions of RNA isolated on DEAE columns (35) and in chains of amino acid acceptor RNA resistant to polynucleotide phosphorylase (26b).

Messenger RNA.—None of the ribosomal and amino acid acceptor RNA's have exhibited any detectable correlation in their base ratios with the DNA composition of the cells from which they are derived. It was postulated, however, that a small portion of the cellular RNA might resemble that of DNA in its composition (49).

Volkin & Astrachan were the first to demonstrate the high turnover of a minor RNA fraction after phage infection in *E. coli* (50, 51). This observation was the starting point of a series of experiments leading to the important result that in *E. coli* (50, 51) and in other bacterial systems (52) this specific RNA fraction apparently mimics the base composition of the infecting phage DNA (considering uracil equivalent to thymine). Nomura, Hall & Spiegelman (53) confirmed the observations of Volkin & Astrachan on the base ratios of the specific RNA synthesized after phage infection. Furthermore, Hall *et al.* (54) demonstrated a specific hybrid formation between denatured

T2-phage DNA and this RNA, which indicates the presence of long nucleotide sequences in the RNA complementary to the T2 DNA. No such hybrid formation is observed with heterologous DNA, even when it has the same over-all base composition as T2 DNA (such as T5 DNA), as would be expected, since such complexes are believed to occur only between strands of complementary base sequences [they have been demonstrated between polydeoxythymidylic and polyadenylic acids (55) and polydeoxyguanylic and polycytidylic acids (56)].

An RNA fraction with similar properties has been found in normally growing organisms. Ycas & Vincent (57) have presented evidence for the existence in yeast of an RNA fraction having a high metabolic turnover and resembling yeast DNA in its base composition. RNA molecules, characterized by active metabolic renewal, have also been detected in normal *E. coli* cells by Gros *et al.* (58). Preliminary data indicate that the base composition of the rapidly turning over RNA molecules in *E. coli*, *Bacillus megatherium*, *Proteus vulgaris*, or *Pseudomonas aeruginosa* (52, 59, 60, 61) is related to the composition of their DNA. Furthermore, the complementarity of the RNA to DNA is indicated by hybrid formation between these RNA molecules and homologous DNA (21, 60). In addition, it has been shown that DNA-RNA hybrid complexes exist naturally in cells actively engaged in protein synthesis [T2 infected *E. coli* (62), *Azotobacter agilis* (63), *Neurospora crassa* (64), *Serratia marcescens* (65)].

The rapidly turning over RNA fraction amounts to only about 3 per cent of the total RNA. Most of this RNA is associated with the ribosomes of the cell, but it differs from the bulk of *E. coli* RNA in electrophoretic mobility and average sedimentation constant (21, 53, 58 to 61, 66, 67), which is around 13S. This RNA is extremely heterogeneous in size. In addition, it is particularly sensitive to degradation in extracts, which probably explains the variability of its reported sizes depending on the method of extraction (66).

A variety of experiments led Jacob & Monod to postulate a new mechanism of information transfer (68): they proposed the existence of a "messenger RNA" having a high metabolic turnover and serving to transfer genetic information from DNA to the sites of protein synthesis, the ribosomes. Thus, ribosomes would be nonspecific and synthesize specific proteins, according to the instructions received from the messenger RNA. This assumption is supported by the observations of Brenner *et al.* (67) that after phage infection no new ribosomes (protein-forming centers) were synthesized. The RNA formed after infection was added to pre-existing ribosomes from which it could be removed by decreasing the Mg^{++} concentration. These observations suggest that phage protein is synthesized by bacterial ribosomes formed before infection, the structural information for phage protein being provided by the RNA fraction discussed above.

This rapid-turnover RNA fraction does, therefore, seem to have all the properties which would be required to allow its identification with the structural messenger:

- (a) It has a base composition reflecting the DNA base composition and possesses entire nucleotide sequences complementary to its homologous DNA.
- (b) It is very heterogeneous with respect to molecular weight, which probably reflects the different sizes of the polypeptide chains to be synthesized.
- (c) It can be associated, under certain conditions, with ribosome sites of protein synthesis.
- (d) Its turnover rate is very high. It has been found that the rate of incorporation of ^{32}P , uracil, and 5-fluorouracil into this fraction is extremely rapid (61).

The mechanism of information transfer proposed by Monod & Jacob is, furthermore, supported by the discovery of Nirenberg *et al.* (69) and subsequent results of Ochoa *et al.* (70) (a variety of homo- and co-polynucleotides, when added to ribosomes, can act as messengers for the formation of specific polypeptide chains) and by the studies on the participation of viral RNA (69, 71) and enzymatically synthesized RNA (72, 73) in amino acid incorporation by a soluble protein-ribosome system from *E. coli*.

Experiments by Chantrenne (74) and Otaka *et al.* (75) indicate that a special type of RNA is accumulated by *Bacillus cereus* in presence of azaguanine. Preliminary experiments (74) suggest that its base composition is related to that of DNA.

So far, the messenger RNA has not been identified with certainty in mammalian cells. As for nuclear RNA and its importance as a precursor of cytoplasmic RNA, it has been discussed in other reviews (1, 2, 76).

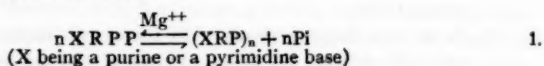
ENZYME SYSTEMS FOR RIBOPOLYNUCLEOTIDE SYNTHESIS

Three main types of reactions have been demonstrated: the first converts nucleoside-5'-diphosphates to form polyribonucleotides with the elimination of P_i ; the second utilizes the ribonucleoside-5'-triphosphates and results in the addition of a limited number of nucleotides to the end of an existing polyribonucleotide with the elimination of inorganic pyrophosphate; the third type of reaction results in the incorporation of nucleotide residues from ribonucleoside-5'-triphosphates into internucleotide linkages of polyribonucleotides. Great progress has been made this year in the elucidation of the mechanism of the latter type of reaction, notably the demonstration of an obligatory requirement for DNA as well as for all four nucleoside triphosphates.

Polymerization of nucleoside-5'-diphosphates.—A complete summary of present knowledge of the purification, properties, and assays of polynucleotide phosphorylase, and the properties of polynucleotides prepared with this enzyme, can be found in recent review articles (2, 3, 77 to 84). In this review, special attention will be given to the mechanism of the enzymatic reaction and the physiological function of polynucleotide phosphorylase.

Polynucleotide phosphorylase catalyzes the synthesis of long chain

polyribonucleotides from nucleoside-5'-diphosphates, with elimination of inorganic phosphate (Eq. 1) (85, 86).



Chemical and enzymic degradation of polynucleotides have shown that they are linear polymers composed of chains of ribonucleotide units linked to one another by 3',5'-phosphodiester bridges. There are two other reactions catalyzed by the enzyme: the phosphorolytic cleavage of polyribonucleotides (the reverse of Eq. 1) and the exchange between ^{32}Pi and the terminal phosphate of the nucleoside diphosphate. Investigations by Heppel *et al.* (83) suggest that a fourth reaction, which would transfer nucleoside monophosphate units from a polynucleotide donor to a polynucleotide acceptor, is also catalyzed by the enzyme.

A single nucleoside diphosphate or mixtures containing two or more diphosphates are polymerized by polynucleotide phosphorylase. However, the rate of synthesis of the copolymer AGUC containing the four species of mononucleotide units (AMP, GMP, UMP, CMP) is considerably slower than the rates of formation of homopolymers (86, 87): the turnover for the enzyme at the purified stage for poly A synthesis with 0.02 M ADP is about 4500 to 5000 moles of nucleotide/min/10⁶g (87). To obtain similar rates, 20 to 30 times more enzyme must be used for the synthesis of poly AGUC from equimolar concentrations of ADP, GDP, UDP, and CDP. This probably is due to the presence of GDP; this nucleotide is polymerized with difficulty and its presence markedly hinders the synthesis of copolymeric polynucleotides (86 to 89).

A. Reaction mechanism of polynucleotide phosphorylase

A better understanding of the reaction mechanisms catalyzed by polynucleotide phosphorylase is not only important in itself, but it very likely would help to clarify the role of this enzyme in cellular metabolism.

The properties of polynucleotide phosphorylase apparently differ in various bacterial species. Enzymes isolated from *E. coli* and *A. agilis** have essentially similar properties, whereas those from *Micrococcus lysodeikticus* and *Clostridium perfringens* might be somewhat different. For this reason, the source of polynucleotide phosphorylase must be considered in any discussion on the mechanism of this enzyme.

1) Primer effects on polymerization

Early experiments by Heppel, Singer & Hilmoe (90) have suggested that polynucleotide phosphorylase catalyzes only the elongation of preformed polyribonucleotide chains and might be unable to catalyze the initiation of new polyribonucleotide chains. It has been demonstrated with preparations of *A. agilis* and *E. coli* that polymer chains are built into pre-

* This organism referred to in the American Type Culture Collection catalog, 5th ed. N9104 (1949) is also called *Azotobacter vinelandii*.

formed oligonucleotides (chain length two or more) when such compounds are supplied in the reaction medium. The oligonucleotides must have free C-3' hydroxyl groups in order to serve as centers for chain proliferation (90).

Highly purified preparations of polynucleotide phosphorylase obtained from *A. agilis* by Ochoa and Mii (87, 91, 92) and partially purified preparations from *M. lysodeikticus* (93, 94) catalyze the polymerization only after an initial lag period, and this lag can be overcome by long chain polyribonucleotides or by oligonucleotides (90 to 95). However, while synthesis of poly AGUC and poly G with the purified enzyme is markedly dependent on the addition of oligo- or polyribonucleotide primers, synthesis of homopolymers (poly A, poly U) can occur rapidly in the absence of added primer (87). Nevertheless, this synthesis can be accelerated by addition of primer when very small amounts of enzyme or Mg^{++} are used. Furthermore, in the case of *M. lysodeikticus*, it was found by Singer *et al.* (93) that, with apparently minor changes in the pH of the reaction medium or the concentration of the substrate, the polymerization of homopolymers is almost completely dependent on oligonucleotide addition.

The effect of primer seems quite clear in the case of the synthesis of poly G (96). Even the crude enzyme cannot begin a poly G chain *de novo*, but has an absolute requirement for an oligonucleotide to which GMP units can then be added. An oligonucleotide with a C-3' phosphomonoester group does not serve as a primer. This is true in the case of GDP polymerization and the polymerization of ADP, UDP or CDP by the *M. lysodeikticus* enzyme (93, 97). On the other hand, these 3' terminally esterified oligonucleotides, like the 3'-hydroxy terminal fragments, can overcome the lag for polymerization of ADP or UDP by *Azotobacter* polynucleotide phosphorylase; however, the 3'-phosphate terminal "primers" are not themselves incorporated into the polymer (90, 95). The ability of these oligonucleotides to overcome the lag is still unexplained. Also unexplained is the mechanism of priming by a polynucleotide. ^{14}C -S-RNA eliminates the lag phase in the polymerization of poly A but is not incorporated into the poly A and is recovered intact from the incubation mixture (41, 98). This raises the very important question as to whether those oligonucleotides incorporated into polynucleotides act as primers by virtue of their ability to serve as a starting point for a new chain, or whether an entirely different mechanism of priming is involved. It was suggested by Beers (94, 97) that the observed stimulation of polymerization by oligonucleotides or polynucleotides is actually a reversal of inhibition of the enzyme by degraded RNA contaminating the enzyme preparation. Some commercial ADP preparations are contaminated by primer oligonucleotides (97). 3'-Phosphate terminal polynucleotides increase, while 3'-hydroxy terminal fragments decrease, in certain conditions, the Michaelis constant of *M. lysodeikticus* enzyme for adenosine diphosphate (97).

Using oligonucleotide primers no specificity with respect to the composition was demonstrable, while priming with polynucleotides shows a certain degree of specificity (81, 87, 91, 92). For example, the synthesis of homopoly-

mers is initiated by the addition of the corresponding, but not the complementary homopolymer. Poly AGUC and RNA prime the synthesis of poly A and U in addition to their own. Poly C is quite exceptional in that it primes the synthesis of every polynucleotide, including poly AGUC and high molecular poly G, whereas only poly C primes its own synthesis. There is no indication that the nature of the added primer has a significant influence in the base composition of the resulting polymer (87). It therefore appears unlikely that a replication or copying mechanism is operative in poly AGUC synthesis, unless such a mechanism is mediated by a polynucleotide contained in the preparation of polynucleotide phosphorylase. The best polynucleotide phosphorylase preparations, purified from extracts of *A. agilis*, contained 3 to 4 per cent of low molecular weight polyribonucleotide with a base composition similar to *Azotobacter* RNA. When poly AGUC is prepared from equimolar concentrations of nucleoside diphosphates, the proportion of nucleotides in the polynucleotide is not equimolar but is closer to that of the polynucleotides contained in the polynucleotide phosphorylase preparation (87, 99). However, this material could simply be a partially degraded RNA contaminant, tenaciously retained by the protein. The relative affinities of the diphosphates for the enzyme may determine the proportion of the nucleotides, in poly AGUC.

It is interesting that the polymerization of ADP by an enzyme purified from *Cl. perfringens* (100, 101) is completely dependent on the presence of basic polypeptides and not of polynucleotides; polyadenylate has no effect. Even in crude *Cl. perfringens* extracts, polyadenylate synthesis is stimulated by various basic polypeptides, such as polylysine, the effect being accompanied by a decrease in the apparent Michaelis constant for ADP from 1.5×10^{-2} M to 5.8×10^{-4} M. ADP polymerization catalyzed by *E. coli* or *A. agilis* polynucleotide phosphorylase is, with certain ratios of ADP/Mg⁺⁺, also stimulated by basic polypeptides (102). If the cell contains basic proteins, it may be that the concentration of diphosphates required for polymerization by polynucleotide phosphorylase *in vivo* would be much smaller than was reported previously (86).

2) Specificity

Crude and partially purified extracts of *Cl. perfringens* catalyze polymer formation from CDP and UDP (100, 101), but these reactions are inhibited by polylysine. After more intensive purification, the enzyme apparently does not polymerize UDP or CDP in presence or absence of polylysine.

This again raises the question as to whether polynucleotide phosphorylase is a single enzyme or a mixture of enzymes, each of which would have an affinity for a definite ribonucleoside diphosphate. Olmsted & Lowe (103) have already reported that on partial purification of polynucleotide phosphorylase from *M. lysodeikticus* (through a procedure which involves trypsin digestion) the enzyme becomes specific for ADP and will no longer catalyze the polymerization of CDP and UDP. They also have some indica-

tions of differences in the sensitivity of the polymerization of various diphosphates to metals. However, Beers (94) has pointed out that the presence of contaminating polynucleotides in a given enzyme preparation might affect the specificity of the enzyme for a particular diphosphate substrate, therefore yielding enzyme preparations with apparently different specificities. The ratio of diphosphate to Mg^{++} is also quite important for polymerization and may vary with each nucleotide. Furthermore, Singer *et al.* (93) using the phosphorolysis assay found no evidence of a separation of activity towards different bases, although the enzyme has now been purified about 300-fold (104). In addition, the polymerization of different diphosphates was equally inhibited by metallic ions. In *E. coli* or *A. agilis* there is no indication of a separation of activities toward the different diphosphates: Throughout the course of purification (500-fold) of polynucleotide phosphorylase from *Azotobacter* extracts (87, 92), the relative rates of exchange of ^{32}P -labeled orthophosphate with ADP, CDP, UDP, GDP, and IDP are essentially constant, suggesting that the same enzyme is acting on all five nucleotides. The specific synthesis of poly AU or poly AGUC by polynucleotide phosphorylase of *A. agilis* or *E. coli* also suggests the action of a single enzyme (87, 105). The presence of more than one type of phosphorylase in these incubation mixtures should lead to the synthesis of the several possible homopolymers as well, and this has never been observed.

Purified preparations of polynucleotide phosphorylase from *A. agilis* can polymerize several other nucleoside diphosphates such as N-methyluridine-5'-pyrophosphate (106), 2-thiouridine-5'-pyrophosphate (107), and 5-fluorouridine-5'-diphosphate (108). Furthermore, contrary to previous reports (99, 109), 5-bromouridine-5'-pyrophosphate is polymerized by polynucleotide phosphorylase, as well as the corresponding chloro- and iodo- compounds (110). Chemical and enzymatic degradation of these polymers show that they possess the normal 3',5' internucleotide linkages; the polymerization of halogeno-uridine diphosphates is in accordance with the fact that polynucleotide phosphorylase polymerizes thymine ribose pyrophosphate (111). Polynucleotide phosphorylase from *E. coli* and *B. cereus* exchanges phosphate with 8-azaguanosine-5'-diphosphate (112). The affinity of polynucleotide phosphorylase for 8-azaguanosine-5'-diphosphate is greater in preparations from *B. cereus* than in those from *E. coli*. It might be significant to recall that in *E. coli* RNA about 10 per cent of the guanine can be replaced by 8-azaguanosine, while in *B. cereus*, as much as 40 per cent of the guanine can be replaced (113).

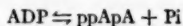
Nucleoside diphosphates esterified at the 3'OH position with PO_4 , such as ppUp or ppAp, are not substrates, but have an affinity for the enzyme, as they inhibit the exchange with the other diphosphates (98). 4,5-Dihydrouridine-5'-pyrophosphate (99, 106) does not act as a substrate in the polymerization or PO_4 exchange reaction, nor does purine riboside-5'-diphosphate (114). 3- β , β -Arabinosyluracil-5'-pyrophosphate (110), adenylyl-5'-methylene-diphosphonate (115), and 6-azauridine-5'-pyrophosphate (116) inhibited

polynucleotide phosphorylase. It appears that polynucleotide phosphorylase can catalyze polymerization of only those ribonucleoside diphosphates which are incorporated *in vivo* into microbial ribonucleic acids (117), with the one exception of bromouracil which does not seem to be incorporated *in vivo* ((118). It should be noted that 5-chlorouracil is incorporated to a small extent into RNA (119). Polynucleotide phosphorylase is inactive when the terminal PO_4 of the ribonucleoside diphosphate is replaced by SO_4 (UMPS or AMPS) (98). Polynucleotide phosphorylase is also specific with respect to the number of phosphates terminally esterified to the nucleoside and to the nature of the sugar moiety of the nucleoside. No reaction occurs with the nucleoside mono- or triphosphates, nor is there any reaction when the ribonucleoside diphosphates are replaced by their corresponding deoxyribose derivatives.

The fact that 6-azauridine-5'-pyrophosphate or ppUp inhibits the ^{32}P orthophosphate exchange in *A. agilis*, when either a pyrimidine or a purine nucleoside diphosphate is used, again suggests that polynucleotide phosphorylase in *A. agilis* is a single enzyme. However, GDP is more sensitive to inhibitors such as thymine deoxyribose pyrophosphate and ppAp than the other diphosphates (98).

3) Phosphorolysis

As in the polymerization, the enzyme is specific with respect to the sugar moiety (DNA is not phosphorolyzed). Singer (120) has demonstrated, in addition, a specificity with regards to the esterification of the terminal phosphate. Tri-, tetra-, and pentanucleotides, which contain a 3'-phospho-monoester end group, are resistant to phosphorolysis. The same holds true for oligonucleotides with 2',3' cyclic phosphate. By contrast, phosphorolysis of oligonucleotides, C-5' terminally esterified or lacking altogether a terminal phosphate, occurs readily, indicating that while C-3' terminal phosphate esters are inhibitors, the C-5' terminally esterified phosphate moiety is not required in order for an oligonucleotide to serve as a substrate. Phosphorolysis of polynucleotides proceeds through a step-wise cleavage, stopping at the dinucleotide or dinucleoside monophosphate level (121, 122). The fact that dinucleotides and dinucleoside monophosphates are not phosphorolyzed suggests that polynucleotide phosphorylase does not catalyze the reversible condensation of two mononucleoside diphosphate units, which is consistent with the requirement for a preformed oligonucleotide in the polymerization reaction. However, if such a condensation occurred, its product might be a dinucleotide containing a pyrophosphate moiety at the terminal C-5' (83) as shown in the reaction:



Data which are obtained with pApA might therefore be misleading, but dinucleotides with a pyrophosphate end, ppApU (prepared synthetically by A. M. Michelson) are not phosphorolyzed either (98). The dinucleotides are therefore not attacked by the enzyme.

The problem of the phosphorolysis of high molecular weight polynucleo-

tides, such as RNA, is more complex. Poly A, U, I, C, G, BrU, IU, CIU, AU, and AGUC are readily phosphorolyzed to completion (42). With the exception of S-RNA, polynucleotide phosphorylase completely phosphorolyzes RNA from several sources (95 to 100 per cent). However, factors such as molecular configuration interfere with the phosphorolysis of the polymers. Under certain conditions, polymers that can assume multi-stranded configurations are resistant to phosphorolysis (42, 123). The interaction between poly A and poly U in aqueous solutions to form helical complexes and the effect of salt concentration on the structure of poly I (24) serve as valuable tools in studying the effect of polynucleotide structure on the rate of phosphorolysis. The results lead to the conclusion that polynucleotide phosphorylase readily phosphorolyzes polymers having a single-stranded nonhydrogen-bonded structure, but acts more slowly on multi-stranded configurations regardless of whether these configurations arise from intra- or intermolecular interactions. This effect of the secondary structure might explain why S-RNA is only phosphorolyzed up to 20 to 50 per cent (26, 27, 42).

4) Exchange

The relationship of the exchange reaction to the mechanism of the polymerization reaction remains obscure. This has been discussed in detail by Grunberg-Manago (77) and Singer *et al.* (93, 121). Although the lag in the polymerization of ADP, UDP, and CDP, noted with highly purified enzyme preparations from *A. agilis*, is overcome by certain oligonucleotides, the exchange reaction (with the same enzyme preparations) is stimulated only slightly by these compounds. It was also found that the exchange can proceed independently from the phosphorolysis reaction. When the ^{32}P exchange reaction with ADP or UDP was studied under optimal conditions—in the presence of their respective polymers—it was found that the total ^{32}P orthophosphate incorporated into the nucleotides was the sum of the incorporation resulting from the exchange, plus that expected from phosphorolysis (121).

Furthermore, while no poly G synthesis by *A. agilis* or *E. coli* occurs in the absence of suitable priming oligonucleotide, the ^{32}P exchange with GDP proceeds readily in the absence of such primers if an optimum GDP/Pi ratio is used (121). The exchange reaction is therefore readily catalyzed by enzyme preparations which catalyze the polymerization reaction with difficulty. However, when poly A is added to ADP or poly U to ADP, the incorporation of ^{32}P into nucleotide material is greatly inhibited (121). Experiments by Ochoa and Mii (81, 91) have shown that poly U inhibits poly A synthesis and vice versa. These results would appear to relate the exchange reaction to the polymerization reaction. The relation was better demonstrated with polynucleotide phosphorylase from *M. lysodeikticus* (93); these preparations differ in several ways from the enzyme preparations from *A. agilis* or *E. coli*. The exchange reaction is greatly stimulated (twenty- to thirtyfold) by the presence of oligonucleotides with unesterified terminal C-3' hydroxyl groups (3'-phosphate terminal oligonucleotides are not active). However, as in the case of the polymerization reactions, the stimulation by

oligonucleotides is dependent on the pH and substrate concentrations; the exchange rate in the absence of oligonucleotides can be increased about twentyfold by minor changes in the pH and substrate concentration with a corresponding decrease in the extent of stimulation by oligonucleotides. The dependence on oligonucleotides and inhibition by polynucleotides for both the exchange and polymerization reactions appears to confirm the assumption that both reactions, in *M. lysodeikticus*, are catalyzed by the same enzyme.

The ^{32}P -XDP exchange is not always indicative of polynucleotide phosphorylase, as for example a highly purified enzyme from yeast catalyzes a rapid exchange between ^{32}P -orthophosphate and the terminal phosphate of several nucleoside diphosphates (41, 98, 124, 125). The yeast enzyme does not phosphorylate any biosynthetic polynucleotide (synthesized by *A. agilis* or *E. coli*), nor does it phosphorylate oligonucleotides. Studies on substrate specificity for the exchange reaction of yeast and bacterial polynucleotide phosphorylase show that the two exchange enzymes are different (98). In contrast to polynucleotide phosphorylase, the yeast enzyme exchanges P when the ribonucleoside diphosphates are replaced by their corresponding deoxyribose derivatives. It is also active with such compounds as UMPS or AMPS and it is probably the same enzyme as the one discussed by Robbins & Lipmann under the name of ADP sulfurlyase (126).

5) Polymers

The availability of high molecular weight polymers has made it possible to obtain considerable insight into the chemistry and the physico-chemical properties of nucleic acids. Studies concerning the interaction of poly A and poly U (106, 127 to 134), and poly I and poly C (135) are in agreement with the theory proposed by Watson and Crick concerning base pairing in a two-stranded helical structure of DNA and were of great help in demonstrating strand separation and specific recombination in DNA (136, 137, 138). These polymers were valuable tools for the investigation of the secondary structure of RNA (139 to 144), its optical properties (145, 146), and the hyperchromicity of polynucleotides (147 to 150). The polymers may also be useful in investigating the mechanism of mutation (139).

The demonstration of formation of hybrid double strands (55, 56) between complementary polyribonucleotides and deoxyribonucleotides stimulates the search for the natural means of information transfer and makes possible the postulation of a mechanism for RNA synthesis involving DNA-RNA hybrid complexes *in vivo*. Furthermore, the use of biosynthetic polymers has allowed an extension of the studies of the specificity and mode of action of nucleases (151) and polynucleotide phosphorylase (42) (see section on phosphorolysis).

Recently, the polymers have proved to be of extreme value in the investigation of the coding system of protein synthesis (69, 70). The fact that they can replace messenger RNA shows that the structure of these biosynthetic polymers is identical to that of RNA.

It should be pointed out that even partially purified polynucleotide phosphorylase preparations are quite suitable for the preparation of polyribonucleotides, particularly for polyadenylic acid, which is less susceptible to nucleases (86, 88, 89, 152, 153).

B. Physiological function of polynucleotide phosphorylase

Of primary importance, however, is the relationship between the reaction catalyzed by polynucleotide phosphorylase and the biosynthesis of the ribonucleic acids by the intact cell. It is not yet apparent how a specific nucleotide composition and sequence in a given species of RNA could be established by an enzyme which appears to polymerize the available nucleoside diphosphates in a relatively random fashion *in vitro* (154). That poly AGUC is a random polymer is also indicated by physical measurements (155). Moreover, the base compositions of poly AGUC reflect substrate concentration rather than primer composition (87, 99). Nevertheless, polynucleotide phosphorylase is found in large quantities in a wide variety of cells: *A. agilis* (85, 86), *E. coli* (156), *M. lysodeikticus* (157), *Alcaligenes faecalis* (158), *B. cereus* (112), *Eubacterium sarcosinogenum* (88), *Cl. perfringens* (100, 101), *Streptococcus faecalis* (159), *Pseudomonas aeruginosa* (160), *Agrobacterium tumefaciens* (89), apparently in liver nuclei (161) and in *Ascaris lumbricoides* (162). This suggests that it has an important function in the metabolism of ribonucleic acid *in vivo*, at least in bacteria. The enzyme could not, however, be detected in soluble extracts of *Lactobacillus arabinosus* (163), although it may be present in other parts of the cell. Indeed, the localization of the enzyme is uncertain. Using a method without regard to the preservation of the ribosomes, polynucleotide phosphorylase is usually found in the soluble portion of the cell, although ribosomes of *E. coli* (164) and *P. aeruginosa* (160) contained some polynucleotide phosphorylase. In *S. faecalis*, however, the enzyme is found in cell membranes (159).

There is a relation between the rate of synthesis of RNA and the activity of polynucleotide phosphorylase of the cell. It has been found with synchronous cultures of *A. agilis* that a parallelism exists between the level of polynucleotide phosphorylase and RNA synthesis during various conditions of growth (165). In a tryptophanless *E. coli* strain there is a correlation between the amount of RNA synthesized and the polynucleotide phosphorylase activity (41, 98, 166). In the absence of protein synthesis (presence of chloramphenicol) they are both a function of the tryptophan added to the medium. Tryptophan can be replaced by DL-5-methyl-tryptophan. The nature of the induced increase of the activity of polynucleotide phosphorylase by amino acids is not clear as yet. There is evidence indicating that, in spite of chloramphenicol, several proteins can be synthesized *de novo* (167). Recent results (168) suggest that polynucleotide phosphorylase is one of these proteins.

It is possible that *in vivo* the enzyme is primarily responsible for the degradation of RNA to yield nucleoside diphosphates and maybe to control the level of inorganic phosphate of the cell. However, with the exception of

tobacco mosaic virus RNA, the rate of phosphorolysis of most RNA preparations is slow (42, 123). S-RNA, in particular, is very slowly phosphorolyzed and only to a small extent. In contrast, messenger RNA is very unstable, and in cell-free extracts of *E. coli* it is degraded to 5'-ribonucleotides (169), suggesting that it is split by polynucleotide phosphorylase. However, the diphosphates have not been isolated.

Assuming that the ribonucleoside diphosphates are the immediate precursors of deoxynucleotides (170), polynucleotide phosphorylase might be the most efficient system for generating DNA precursors from RNA and eliminating the information contained in messenger RNA. Indeed, it has already been shown (171) that prelabeled components of viral-induced RNA disappear from this fraction and appear in phage DNA.

In order to investigate the role of polynucleotide phosphorylase *in vivo*, a method for measuring the activity of the enzyme within the cell has to be found. Polynucleotide phosphorylase can be reliably assayed in suspensions of *E. coli* exposed to toluene (166) and in crude *E. coli* extracts (168) both by the ADP-¹⁴C incorporation assay (156) and by measurement of the phosphorolysis of poly A (87). There is some question concerning the validity of the ³²P-exchange assay (77) (see section on exchange). The synthesis of polynucleotides has also been carried out in coacervate drops made of RNA and histone (172).

Incorporation into a terminal or sub-terminal position of ribonucleic acid.—Earlier work demonstrating the existence of a mechanism for the addition of a few nucleotide residues to a pre-existing RNA chain has been summarized previously (38, 173 to 177). It has been shown that only nucleoside triphosphates are substrates. An amino acid acceptor RNA can accept the nucleotide residues, and these appear to be added in specific sequences to the end of the RNA (177, 178). Two cytidylate units are incorporated per one adenylate (177), suggesting that the product is RNA pCpCpA. The enzyme system responsible for this incorporation has been purified several hundredfold from the soluble cytoplasm of rat liver (38, 39, 175, 176, 178 to 182) and from *E. coli* (183, 184). The two systems incorporate AMP and CMP from ATP and CTP. The constant ratio of the rate of incorporation of AMP to that of CMP throughout the purification of the enzyme from *E. coli* suggests that a single enzyme is responsible for the incorporation of both nucleotides. A definite conclusion must, however, await a more extensive purification. Nucleoside mono- or diphosphates or the corresponding deoxynucleoside triphosphates do not serve as nucleotide donors. The incorporation of each nucleotide unit is accompanied by the liberation of one equivalent of inorganic pyrophosphate. It requires the presence of Mg⁺⁺ and a preparation of amino acid acceptor RNA from which the terminal nucleotides have been removed, either by a partial digestion with snake venom or by pyrophosphorolysis (see later). Depending upon the extent of prior degradation of the RNA, either AMP or AMP plus one or two CMP residues is

incorporated. Alkaline degradation studies show that the two CMP residues are adjacent to each other and that AMP is linked to the terminal cytosine phosphate, confirming that the terminal trinucleotide sequence of the amino acid acceptor RNA of *E. coli* is analogous to that previously mentioned for RNA of mammalian cells. *E. coli* enzyme also catalyzes a net pyrophosphorolysis of the intact amino acid acceptor RNA, yielding ATP and CTP from the terminal trinucleotide, but no UTP or GTP (183, 184). The enzyme removes the same residues which it incorporates. Intact or partially degraded ribosomal RNA from *E. coli* is not pyrophosphorolyzed, nor does it function as an acceptor of CMP from CTP (183); synthetic polynucleotides A, C, AGUC, and AU, as well as several plant virus RNA samples (tobacco mosaic virus and turnip yellow mosaic virus), fail to function as acceptor with the *E. coli* enzyme (25). *B. cereus* RNA and ribosomal rat liver RNA were inactive as nucleotide acceptors with the liver enzyme (39). Even acceptor RNA itself, after removal of somewhat more than the pCpCpA segment or after being subjected to relatively few internal diester cleavages, is inactive as a nucleotide acceptor or as a substrate for the pyrophosphorolysis (25).

If it is a single enzyme which incorporates both the AMP and CMP in the very precise order of 2 CMP residues and 1 AMP residue, the specificity of this enzyme is quite remarkable. It can add CMP only to the pre-existing fourth nucleotide or to some sequence or configuration involving the fourth nucleotide. This fourth nucleotide contains adenine in 69 per cent of the *E. coli* RNA chains (25) and in most of the rat liver amino acid acceptor RNA (182). The enzyme, therefore, can distinguish between an adenine which is in the fourth position and one which is at the end of the sequence pCpCpA.

Studies by Canellakis & Herbert (175), Hecht *et al.* (177b), and Harbers & Heidelberger (185) have suggested that not only CMP and AMP, but also GMP and UMP can be linked to the ends of pre-existing RNA chains. The significance of RNA chains terminated by pUpUpG or pUpU for amino acid acceptor activity is, however, obscure.

Also obscure is the function of several incorporating enzymes specific for one ribonucleotide. An enzyme purified from extracts of ascites cells (186) incorporates UTP into the terminal end of RNA. Ribosomal RNA can act as nucleotide acceptor; the incorporation of UMP is stimulated by a mixture of amino acids. An enzyme purified from calf thymus gland (187, 188) incorporates CTP into the terminal position of thymus RNA in the sequence . . . pCpC. This enzyme is highly specific for CTP and, in contrast to the previous CTP-incorporating enzymes discussed, is inactive with ATP.

Separate enzymes may therefore be required for addition of each ribonucleotide. It is not known whether these incorporating enzymes are part of a group of similar enzymes involved in the complete synthesis of RNA chains or whether they serve to regenerate the terminal grouping of RNA.

Synthesis of polyribonucleotides requiring nucleoside triphosphates.— Since the earlier work showing that mammalian preparations can catalyze the incorporation of ATP into internucleotide linkages of polyribonucleotides (189 to 192) many systems have been found utilizing the nucleoside triphosphates for the incorporation of nucleotides into RNA. They can be separated into two groups, depending on whether the synthesis requires the presence of DNA or not; some of the systems not requiring DNA are enhanced by RNA.

A. DNA-dependent RNA synthesis

Weiss *et al.* (193) first isolated from rat liver a particulate enzyme (RNA polymerase) which requires the presence of all four triphosphates for the incorporation of any one into RNA. Preincubation of the enzyme with small quantities of DNase inactivated the system, suggesting that DNA is involved in this RNA synthesis. Similar RNA polymerases have been found in *M. lysodeikticus* (194), *E. coli* (195 to 200), *L. arabinosus* and *A. agilis* (163; 201), thymus nuclei (188), rabbit reticulocytes (202), and in plants (pea seedlings) (203).

1) Nature of the product

The radioactive nucleotides ATP, GTP, CTP, and UTP are incorporated into a product which is acid insoluble, nondialyzable, resistant to DNase, and sensitive to RNase and alkaline hydrolysis. When RNA, prepared with any one of the nucleoside triphosphates labeled with ^{32}P in the α -phosphate, was hydrolyzed with alkali, all of the 2',3'-nucleotides released were labeled. This indicates that labeled nucleotide residues are linked to the other three monophosphates. The combined action of phosphodiesterase and phosphomonoesterase (from snake venom) releases P_i and $^{32}\text{P}_i$ at about the same rate, which is a further proof that the incorporated nucleotides are predominantly nonterminal and distributed throughout the RNA chains (163, 193). Alkaline hydrolysis of RNA prepared from ^{14}C -ATP liberated almost quantitatively 2',3'-AMP and no adenosine, confirming that the incorporation is not terminal (163).

With a partially purified enzyme (100- to 300-fold) from extracts of *E. coli* (198 to 200) or extracts of *M. lysodeikticus* (194a), a two- to eightfold increase of polyribonucleotide can be demonstrated by the increase in absorbancy at 260 $\text{m}\mu$ or in orcinol reacting material.

2) Requirements

The incorporation of nucleotide is markedly dependent upon the presence of all four nucleoside triphosphates (substitution by the corresponding diphosphates or deoxyribonucleoside triphosphates results in a marked reduction of isotope incorporated). A reaction in which ATP is converted into an acid-insoluble form in the absence of the other three triphosphates will be described in a later section. An -SH compound (omission of β -mercapto-ethanol resulted in a 50 per cent loss (200)) and a divalent cation such as Mg^{++} (198 to 201) or Mn^{++} (194a) are also required; Mg^{++} is more efficient in some cases, Mn^{++} in others, while for *E. coli* enzyme maximal

rates occur only when both are present (198, 200). The purified enzyme has an absolute requirement for DNA from any one of several sources.

The affinity constant of the substrates for the enzyme are small, the K , values for the triphosphates are between 10^{-5} and 10^{-4} M, and 0.28 O.D. units for thymus DNA (198, 200). There is a relationship between the amount of nucleotide incorporated and the release of inorganic pyrophosphate. The requirements for the release of inorganic pyrophosphate and for the incorporation of triphosphates are the same, indicating that pyrophosphate is produced by the same enzyme which incorporates the triphosphates (198). Optimum pH is between 7.5 and 8 (194, 198 to 200). Pyrophosphate inhibits the reaction, whereas inorganic phosphate has no effect.

3) Role of DNA

DNA's isolated from many sources and having widely different compositions are active (193 to 204) while RNA, including tobacco mosaic virus RNA (198), cannot be substituted for DNA. The correspondence between the nearest neighbor sequence and base composition (194, 196, 198, 199c, 200, 204) of the synthesized RNA and the primer DNA demonstrates that DNA is directly involved in the reaction and does not function indirectly, merely to inhibit some nucleases. Thus, the base ratio adenine + thymine/cytosine + guanine in the primer DNA preparations used, which ranged from 0.4 to 1.86, is closely reflected in the pattern of incorporation of ribonucleotides into the product. Also, the sum of the purines incorporated into RNA is equal to the sum of pyrimidines. The nearest neighbor sequence (204) following the method used by Josse *et al.* (205) for DNA has been determined using four different DNA's as primers: *M. lysis-deikticus* DNA, which has a relatively high guanine and cytosine content, T2 phage DNA rich in adenine and thymine, *E. coli* DNA, and calf thymus DNA. The results indicate that the frequency of the 16 nucleotide pairs from primer and product is remarkably close.

The similarity in the nearest neighbor frequency and in the base composition of DNA and RNA provide evidence for the assembly of ribonucleotides along a DNA template. How DNA transfers its sequence information to RNA is not entirely clear. One possible mechanism is that incorporation of ribonucleotides is determined by the ability of the new polymer to form hydrogen-bonded pairs with the bases in the induced DNA, as proposed by the Watson and Crick model for DNA itself. It is particularly significant that the base composition of the RNA formed in the presence of the single-stranded ϕ X174 DNA (206) is complementary to that of this DNA (198, 200). This result has no ambiguity due to complementarity of the two DNA strands. Further support of this hypothesis was obtained by using the bio-synthetic poly dT homopolymer (196), the dAT copolymer (197, 198), and dGdC polymers (200) as primers. If poly dT is used as primer, only AMP is incorporated, either in the presence or absence of the other nucleoside triphosphates. The product of the poly dT initiated system has been further

characterized as poly A by alkaline degradation of both ^{14}C - and ^{32}P -labeled material. In addition, the presence of RNase is without effect, which is consistent with the resistance of poly A to RNase. In the presence of dAT copolymer, AMP incorporation is dependent on the presence of UTP, and UMP incorporation is dependent on the presence of ATP, while GMP and CMP are not incorporated and their addition has no appreciable effect. Only GMP and CMP are incorporated in the presence of dGdC. Experiments with labeled ATP or UPT show that the polymer made by using AT copolymer as primer (197, 198) is composed of alternating units of adenylic and uridylic acid, therefore mimicking the dAT copolymer (207). These results confirm that the DNA primer determines not only the composition, but also the sequence of the ribonucleotides in the RNA product.

The fact that RNA synthesized by the RNA polymerase is a copy of its initiator DNA is further suggested by the ability of T_2 DNA to form specific complexes with the RNA synthesized in its presence (208).

An ambiguity exists in the relative efficiency of single- (as compared to double-) stranded DNA for priming of polynucleotide synthesis. In general, DNA preparations are less active after heat denaturation (201). However, results of Hurwitz (197, 198) using the biosynthetic polymer dT and ϕX174 DNA indicate that single-stranded DNA can prime RNA synthesis. The greater susceptibility of single-stranded DNA to nucleases (209) might explain the lowered activity of the heated DNA.

The similar base ratios found for RNA and its primer DNA and the similarity in nearest neighbor frequencies are consistent with two possibilities: (a) both DNA strands are employed as templates for RNA; (b) the over-all base composition of the two complementary DNA strands is alike and one or both may prime. In the latter case the results offer no information on the mechanism of priming.

Using the double-stranded form of ϕX174 DNA, in which it is known that the base composition of the two strands differ, it was found that both strands serve to direct the synthesis of RNA (200). That both strands of DNA are capable of serving as a template is also indicated by an experiment in which all the primer DNA was converted into a hybrid after mixing with the RNA product (208). This may not occur *in vivo* with DNA as its exists in the genome. Some structural feature in the chromosomal DNA may cause RNA synthesis to copy the sequence of only one of the two strands.

An intermediate stage in the mechanism of DNA action may involve the formation of DNA-RNA hybrid molecules. Such complexes have been shown to exist in nature (see section on messenger RNA). It might be significant that a hybrid complex is considerably more stable than one in which both strands are of the deoxyribose type, as in DNA (56). Preliminary results (198) suggest that the reaction product of the RNA polymerase consists of an RNA-DNA hybrid complex; indeed, some of the labeled product is resistant to dilute RNase and DNase and becomes sensitive to RNase on acid treatment. However, Weiss, Geiduschek *et al.* (208), using *M. lysodeikticus*

enzyme, were unable to find such complexes. Furthermore, the buoyant density of phage DNA does not change before and after serving as an initiator and this might indicate that it is the double helix form of DNA which acts as a template, or that the double helices do not have to unwind entirely to serve as an RNA template (a double helix in which only a small portion of the nucleotide sequence is maintained in register can rewind in such a manner as to obliterate, for the purposes of the density gradient experiment, all traces of its unfolding) (210, 211).

While the need of DNA is demonstrated, the possibility of an additional requirement for RNA in the synthesis of RNA by RNA polymerase has not been ruled out as the enzyme preparations are contaminated by RNA. However, RNase addition does not affect AMP incorporation when poly T is used as a primer, while DNase addition is inhibitory (196, 198). Further evidence has been obtained by studying the end group of the polyriboadenylate product formed from ^{14}C -ATP with poly T as inducer. The presence of radioactivity on the 5'-nucleoside diphosphate from the P terminal end of the chain suggests that the synthesis of poly A begins, at least in part, *de novo* with the added labeled nucleotide. However, the amount of nucleoside liberated after alkaline hydrolysis is larger than the amount of diphosphate formed. It is suggested by the author that this discrepancy might be due to the addition of ribonucleotide to the end of DNA chains (198).

4) Formation of polyadenylic acid

Purified fractions of RNA polymerase (199b, 200) catalyze the conversion of ^{14}C -ATP to an acid-insoluble form in the absence of the other three ribonucleoside triphosphates. There is no incorporation of CMP and GMP when the corresponding nucleoside triphosphates are added singly to the reaction, although UMP incorporation occurs to a small but significant extent (200). Neither RNA, nor polyadenylic acid, nor dAT copolymer replaces the DNA requirement. DNA is required not only for the initiation of polyadenylic synthesis, but also for the continued formation of the polynucleotide. One explanation (200) of the priming effect of DNA would be to assume a sequence of thymidylate residues in the DNA. The addition of other ribonucleoside triphosphates results in an inhibition of polyadenylate synthesis. The question of whether polyadenylic acid synthesis is catalyzed by RNA polymerase or by another enzyme contaminating the polymer cannot be answered at the present time.

It is suggested (212) that not only messenger DNA-like RNA but also amino acid acceptor RNA is synthesized by a system requiring DNA. However, it is difficult to rule out that the isolated amino acid acceptor RNA is not contaminated with messenger RNA.

Another enzymatic reaction should be mentioned which has the possibility of relating RNA synthesis to DNA: enzyme preparations isolated either from *E. coli* (213) or associated with calf thymus nuclei (214 to 217) incorporate any one of the four ribonucleoside triphosphates into an insoluble

form which was rendered acid-soluble by DNase treatment. Investigation of this system has led to the finding that a ribonucleotide as such is incorporated into a polynucleotide chain with a typical diester linkage formed between it and adjacent deoxynucleotides. The *E. coli* system (213) in order to incorporate CTP requires the other three deoxynucleoside triphosphates and DNA. Controls, lacking a single deoxynucleoside triphosphate, show a negligible incorporation of isotope. Addition of DNA polymerase (218) results in a marked stimulation of CTP incorporation.

The thymus enzyme differs in certain significant respects from the *E. coli* enzyme. It also differs from the DNA polymerase. It does not require the presence of the complementary deoxyribonucleoside triphosphates. In addition, the extent of incorporation is not affected by the removal of a large portion of the DNA polymerase activity. Finally, the thymus enzyme can incorporate only a limited number of deoxyribonucleotides or ribonucleotides into DNA. It predominantly incorporates ribonucleotides or deoxyribonucleotides into terminal positions of the DNA. Any one of the ribo- or deoxynucleoside triphosphates can be used for this limited incorporation (215, 216, 217). The DNA containing a single ribonucleotide at the terminal C-3' position (ribonucleotidyl DNA) can act as an acceptor for ribonucleotides when incubated with enzymes which normally incorporate ribonucleotides into RNA (219, 220).

The significance of these enzymes to RNA synthesis and their relationship to the polymerase action are not clear as yet. A comparative study of the properties of these systems will be necessary.

B. RNA-dependent RNA synthesis

An enzyme specific for the incorporation of ATP has been purified from extracts of calf thymus nuclei (188, 221, 222). The purified enzyme catalyzes the formation of a polyriboadenylate more than 100 units long. Its activity is dependent on a polyribonucleotide which appears to contain an adenylate sequence. Polyadenylic acid synthesized by polynucleotide phosphorylase can function as an active primer.

An apparently similar enzyme, but specific for UMP incorporation, has been isolated from pigeon liver microsomes (223) and rat liver ribosomes (224, 225). The enzyme catalyzes the formation of polyuridylic acid. It is specific for UTP, being less active with CTP, and incorporating only traces of ^{14}C -AMP and ^{14}C -GMP. The enzyme does not seem to require a specific polyribonucleotide chain as a primer since ribosomal RNA or ribonucleotidyl DNA is active. The function of the primer is probably to provide sites of attachment for polyuridylylate chains.

A soluble enzyme isolated from Ehrlich ascites carcinoma cells incorporates labeled UMP from UTP to RNA (186, 226, 227). The UMP residues are located in terminal and nonterminal positions of the polynucleotide chain. Fractionation of the extracts yields two fractions: one catalyzes the incorporation of only a limited amount of UTP into the terminal positions of the existing chains (this was discussed in a previous section); with the

other, the incorporation of UTP is greatly increased by the addition of a mixture of 5'-triphosphates—ATP, GTP, and CTP—and most of the incorporated radioactivity is distributed in internucleotide linkages. Addition of purified RNA from whole ascites cells enhances this incorporation. The fraction also exhibits a requirement for DNA. It was shown by using ^{32}P -UTP that in the absence of the three triphosphates most of the uridine residues in the polynucleotide are adjacent to one another, while in the presence of the other triphosphates more random distribution of the incorporated uridine residues occurs. Further purification will tell if one or more systems are present in this fraction.

An enzyme purified from *E. coli* particles incorporates ATP into RNA (198, 220, 228). The purified enzyme catalyzes the formation of a polyribadenylate. Ribosomal RNA or ribonucleotidyl DNA can act as a primer (220). The partially purified enzyme preparation of Hurwitz *et al.* (198) catalyzes the incorporation of all four ribonucleotides if they are present singly. When all four nucleoside triphosphates are present, incorporation of the labeled nucleotide is inhibited. The enzyme preparation of Gottesman *et al.* (220) is most active with ATP, although it will incorporate CMP and UMP into polynucleotide form at much lower rates. GTP is not a substrate for this enzyme.

An enzyme, polynucleotide synthetase (229) purified from the soluble cytoplasmic fraction of spinach leaves, catalyzes the incorporation of the label of ATP- ^{14}C into RNA. In contrast to the other RNA primer systems, maximal incorporation is obtained in the presence of all four ribonucleoside-5'-triphosphates—ATP, GTP, UTP, and CTP. Magnesium ions and RNA are essential requirements. However, RNA has to be combined with protein of the enzyme preparation prior to its addition to the reaction mixture. DNA cannot be substituted for RNA and the incorporation of ATP- ^{14}C is not affected by the presence of deoxyribonuclease.

In conclusion, more than one enzymatic system exists for RNA synthesis. Even a homopolymer like polyadenylic acid can be synthesized by at least three different mechanisms: (a) from diphosphates by polynucleotide phosphorylase; (b) from triphosphates by a system requiring deoxypolynucleotides as primer; and (c) from triphosphates by a system requiring ribopolynucleotides as primer.

The enzymes catalyzing RNA synthesis may be broadly separated into two groups: One group catalyzes reactions forming polynucleotides whose composition is primarily determined by the type of nucleic acid primer added, such as RNA polymerase which is probably responsible for messenger RNA synthesis *in vivo*. The enzyme catalyzing the terminal addition of a pCpCpA group to RNA might be included in this group, as it is highly specific for amino acid acceptor RNA. Its apparent role is to restore the terminal end of amino acid acceptor RNA to its functional form.

In the other group, the composition of the polynucleotides synthesized is

not greatly affected by the nature of the primer added to the reaction. In this group are polynucleotide phosphorylase where the primer effect is not understood as yet, as well as enzymes which presumably form polynucleotide addition products on the primer RNA and which exhibit a narrower specificity with respect to nucleotide substrate. The function of these enzymes *in vivo* is not as yet understood.

DEOXYPOLYRIBONUCLEOTIDE SYNTHESIS

The enzymatic synthesis of DNA has been discussed in several recent reviews (2, 3, 230, 231, 232) and will only be briefly described here.

SYNTHESIS OF DNA

The only known reaction for deoxyribopolynucleotide synthesis is the one catalyzed by the polymerase enzyme discovered by Kornberg *et al.* (218, 233). The reaction involves Mg^{++} and all four deoxyribonucleoside triphosphates (adenine, guanine, cytosine, and thymine) and has an absolute requirement for a primer DNA. It has not yet been possible to synthesize a DNA having a transforming activity, but studies (234 to 241) on the enzymatic pathways specifically developed in response to a given virus infection strongly suggest that DNA in the living cell is synthesized by a polymerase type of reaction (see also reviews) (1 to 3, 231, 242). In spite of the fact that the enzyme has been purified 2000- to 4000-fold (232), sufficient nuclease might be present in the preparation to destroy the biological activity of the synthesized DNA. The polymerase system, discovered in *E. coli*, has been shown to occur in mammalian cells (243) and has been purified from extracts of calf thymus (244), regenerating rat liver (245), and Ehrlich ascites cells (246). Some differences between mammalian and *E. coli* polymerase exist, particularly with respect to the primer. There is also some difference between the purified polymerase of uninfected *E. coli* and that synthesized in *E. coli* after T2 infection (247), which leads to the conclusion that bacteriophage infection results in a synthesis of a new polymerase.

The enzymatically synthesized DNA is indistinguishable from initiator DNA in terms of viscosity, sedimentation coefficient, molecular weight, and composition. The ratios of the number of adenine-thymine pairs to the number of guanine-cytosine pairs is the same in the synthesized and initiator DNA, even with widely different concentrations of the four substrates and with net increases in DNA varying from 2 to 1000 per cent (232). Furthermore, studies of the dinucleotide frequencies (205) that are produced in the newly synthesized DNA with different DNA's as initiators indicate that: (a) The frequencies are unique with each DNA and reflect nonrandom ordering of the nucleotides in the DNA chains; (b) the newly synthesized DNA has the same nearest neighbor frequencies whether the primer is native DNA, heat-denatured DNA, or enzymatically prepared DNA containing only traces of the original native DNA; (c) the relative amounts of certain dinucleotide frequencies are consistent with the Watson and Crick

model of hydrogen bonding and strands of opposite polarity. Therefore, the enzymic replication involves base pairing of adenine to thymine and of guanine to cytosine in two strands of opposite direction.

The results of the nearest neighbor frequency analysis provide the clearest evidence for the view that the enzymatic synthesis of DNA involves the sequential ordering of nucleotides along a DNA template, on the basis of hydrogen bonding of adenine to thymine and of guanine to cytosine. Consistent with this is the observation that specific replacement of certain bases by analogues can be made, provided that the correct hydrogen-bonding relations are maintained (248): Thus, uracil and 5-bromouracil can replace thymine. The absence of uracil from ordinary DNA may be explained by the fact that extracts of *E. coli* contain a specific and very potent dUTPase (249).

The question still remains as to whether or not a separation of the two DNA strands is necessary for replication. Bollum observes (250, 251) that, whereas calf thymus polymerase will use single-stranded DNA (such as ϕ X174 DNA, or chemically or physically disrupted DNA) as primer, it cannot use double-stranded DNA, even after degradation by chain scission to low molecular weight products. This result supports the assumption that single strands or single-stranded regions are the actual priming site for deoxypolynucleotide synthesis.

This also appears to be the case with the polymerase of *E. coli* formed after bacteriophage infection and able to utilize certain DNA's as primer only if they are heated (247). There is also suggestive evidence implicating denatured DNA as a priming entity in noninfected bacterial system. Treatment of DNA with minute amounts of DNase or by heating results in a two- or threefold increase in priming ability. Furthermore, the single-stranded ϕ X 174 DNA is a better primer than double-stranded calf thymus DNA (232). In all cases the denatured DNA molecule will initiate synthesis of the organized hydrogen-bonded structure characteristic of most native DNA's. The decision, however, as to whether disruption, or at least some modification, of the macromolecular organization of the primer is essential for DNA synthesis in bacterial systems must await the availability of an enzyme preparation that is completely free of nuclease activity.

If primer DNA is actually single-stranded DNA and if the synthetic reaction produced double-stranded molecules, then the reaction should cease when the primer DNA has been doubled in amount. Increases of more than twofold should be possible only with enzyme systems capable of converting native two-stranded nonpriming DNA into denatured single-stranded DNA. It remains to be seen whether such a reaction limit exists.

Another problem which is not solved is whether the polymerase can start a chain *de novo* or whether covalent binding between the primer DNA and the replicating chain is necessary.

In relation to this, it has been reported (252) that a single nucleoside triphosphate can be linked to the deoxynucleoside end of DNA by *E. coli* polymerase, and it has been shown that the deoxynucleotide is covalently

linked to the DNA molecule. Any one of the four deoxyribonucleoside triphosphates is active in this reaction.

With calf thymus polymerase any one of the deoxynucleoside triphosphates can also be incorporated in the presence of thymidylate oligonucleotides as primers (253). Thymus polymerase (251), in contrast to *E. coli* polymerase, can utilize as primer small oligodeoxyribonucleotides, acid-soluble deoxyribopolynucleotides from a pancreatic digest of DNA, or chemically synthesized thymidylate polymers containing 3 to 7 thymidylate residues (254). With thymidine triphosphate or deoxyguanosine triphosphate in the presence of thymidylate oligonucleotides (253), a series of compounds are formed which represent an addition of one, two, three, or more units of deoxynucleotides to the primer. However, with dAPP or dCPPP as substrate in the presence of identical polythymidylate primers a single product is formed which can be separated from the polythymidylate primer. It would appear therefore that the priming action of oligonucleotides does not involve hydrogen bonding.

Further work is required to elucidate the relation between these latter reactions of single deoxynucleoside triphosphate and the extensive DNA synthesis observed when all four nucleoside triphosphates are present.

SYNTHESIS OF DEOXYNUCLEOTIDE POLYMERS

In the absence of added primer and after two to five hours lag period, *E. coli* polymerase catalyzes the polymerization of deoxyadenosine triphosphate and deoxythymidine triphosphate to form a copolymer of deoxyadenylate and deoxythymidylate (dAT copolymer). The polymer contains equal amounts of deoxyadenylate and deoxythymidylate in perfectly alternating sequence (207).

Even though dAT copolymer arises without a primer, it is nevertheless a specific molecule with the properties of a double helix: Physical measurements indicate that the dAT copolymer, like DNA, is a double-stranded structure involving hydrogen bonding between the adenine residues in one chain and the thymine moiety in the other, with a molecular weight of several million. However, it is very susceptible to nuclease activity. In particular it is degraded (205) by Lehman's *E. coli* phosphodiesterase (209), an enzyme which only attacks heated DNA and single-stranded ϕ X174 DNA, but not native double-stranded DNA.

Isolated dAT copolymer primes the enzymatic synthesis of an identical polymer without any lag. Even in the absence of primer, the size of the polymer formed remains constant throughout the polymerization process. This suggests that the reaction is a rapid replication process, following an initial *de novo* phase during which the first few polymer molecules are synthesized.

E. coli polymerase also catalyzes the polymerization of deoxycytidylate and deoxyguanylate (255) after a lag phase; this polymer, as opposed to the dAT copolymer, consists of two homopolymers, polydeoxyguanylate and

polydeoxycytidylate, in unequal amounts. Indeed the dGdC polymers prime not only their own synthesis without any lag, but also the incorporation of deoxyguanylate or deoxycytidylate alone.

The mechanism for achieving perfect alternation remains unknown, as does the significance of the prolonged lag period preceding the initiation of synthesis. One might speculate that it is during this interval that the appropriate primer is somehow formed.

The deoxynucleotide polymers have been particularly useful for the investigation of information transfer.

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PROTEIN BIOSYNTHESIS^{1,2,3}

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The past year has witnessed a number of exciting developments which offer great promise toward an eventual complete understanding of the final steps involved in protein biosynthesis. Among these developments has been the accumulation of evidence of the existence of a new type of RNA which may possess some of the postulated properties of the long-mythical template. Ribosomal systems have been found to respond specifically to the addition of synthetic polynucleotides whose nucleotide composition appears to specify the amino acids which are incorporated into peptide linkage. These experiments, along with the amino acid replacements which have been observed in chemically produced mutants of tobacco mosaic virus, have provided an experimental approach toward the deciphering of the RNA-protein "code." The purification of the enzyme which transfers amino acyl sRNA to ribosomal protein supplies the chemical approach necessary to make use of the above information.

These topics are among the subjects treated in this review, but apologies are offered for the large number of important topics which perforce had to be omitted from discussion. The reader is referred to recent reviews on nucleic acid structure, metabolism, and synthesis in this volume (1, 2) or in recent companion volumes (3, 4, 5, 6, 7), reviews on virus and phage biochemistry (8, 9), on the biosynthesis of amylase by pancreas extracts (10), on the biosynthesis of serum albumin (11) and of hemoglobin (12) by microsomal systems, on protein synthesis in nuclei (13, 14), on biochemical genetics (15, 16), on ribosomal proteins (17), on general aspects of protein synthesis (7, 18, 19,

¹ The survey of most of the literature pertaining to this review was concluded in November 1961.

² The following abbreviations are used: sRNA (soluble, transfer, acceptor, or shuttle RNA); mRNA ("messenger" or "informational" RNA); AMP (adenosine-5'-monophosphate); CTP, GTP, UTP (the triphosphates of cytosine, guanosine, and uridine); A, C, T, U (adenylic, cytidylic, thymidylc, and uridylic acid ribonucleotides when present in polynucleotides); poly I (polyinosinic acid); PP (pyrophosphate); RNase and DNase (ribonuclease and deoxyribonuclease); DEAE (diethylaminoethyl); EDTA (ethylenediaminetetraacetic acid); NAD (nicotinamide-adenine dinucleotide); TCA (trichloroacetic acid); TMV (tobacco mosaic virus.)

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20), on protein and nucleic acid synthesis in cell cultures (5), and on nucleotide-peptide compounds (2, 21).

THE ACTIVATION OF AMINO ACIDS

The mechanism of the activation of amino acids for protein synthesis is now well established (7). As in the activation of acetate, fatty acids, and pantoic acid, an enzyme-bound acyl adenylate is formed in which the carboxyl group of the amino acid and the phosphate of 5'-AMP are bound in a mixed anhydride linkage. The transfer of the amino acid from this enzyme-bound intermediate to the 2' or 3' position of the terminal adenosine residue of a soluble RNA (sRNA)—a reaction in which an ester linkage is formed—is formally analogous to the acyl transfer reaction in which acetyl adenylate is converted to the thioester, acetyl CoA. Indeed, the negative free energy of hydrolysis of both types of ester is high, and evidence is beginning to accumulate that the sRNA functions catalytically in acyl-group transfer in a manner analogous to coenzyme A, shuttling back and forth between soluble amino acid-activating enzymes and the ribosomal particle, and transferring its bound amino acyl group to the latter (see later discussion). Soluble RNA has also been referred to as transfer RNA and acceptor RNA; the future will decide whether shuttle RNA (sRNA) may not prove a more appropriate term.

Amino acid specificity.—The degree of specificity of the activating enzymes toward amino acids is of interest, particularly in relation to the question of how the cell avoids making "mistakes" in specifying the final order of amino acids in the protein. For example, should sRNA play the role of the postulated adaptor (24, 117) in the assembly of amino acids on a template, a mechanism in which the chemical identity of the amino acyl group might be completely subordinated to that of the sRNA, it would be of primary importance to the cell to discriminate between amino acids as finely as possible in the steps leading to the formation of the amino acyl RNA. Fairly high discrimination in each of these two steps could result in very high amino acid specificity in the formation of amino acyl sRNA, and would circumvent the difficult requirement that absolute specificity reside in a single step. It should be noted, in this connection, that the degree of discrimination of the overall mechanism must be sufficient not only to reject 19 unwanted protein amino acids, but also some of the many nonprotein amino acids which are found in nature.

The conclusion which may be reached from earlier studies (7), including the more recent systematic studies of Bergmann *et al.* (25), is that the specificity of the activating enzymes, while high, is not absolute. The latter studies in which purified *Escherichia coli* enzymes were used, show that isoleucine-activating enzyme also catalyzes the formation of L-valyl adenylate, and that the valine enzyme also activates L-threonine. DL-Allothreonine, however, is not acted on by the latter enzyme although its Cl analogue, DL- α -amino- β -

chlorobutyrate, is activated at a rate about equal to that of DL-valine, and possesses a K_m value only slightly greater. However, the K_m values for valine and the isoleucine enzyme and for threonine and the valine enzyme are two orders of magnitude higher than for the "normal" substrates, and the maximum velocities are considerably lower as well. It is, therefore, unlikely that this relative specificity could result in an appreciable number of "mistakes" in amino acid sequence. Loftfield & Eigner (26) have also studied these *E. coli* enzymes and have found that valine activation by the valine-activating enzyme is inhibited by isoleucine, leucine, alloisoleucine, and α -aminobutyric acid, while valine activation by the isoleucine enzyme is inhibited by isoleucine. These results offer a possible explanation for the well-known inhibition of protein synthesis by dietary amino acid imbalances.

In general, those amino acid analogues which can be incorporated into protein are activated by the appropriate activating enzyme, and those which do not become incorporated are not activated and sometimes act as competitive inhibitors. This subject has been reviewed recently (7, 22, 23) and it is only necessary to add that, in contrast to the previous finding (27) that ethionine is not activated by the methionine-activating enzyme from rat liver (a tissue which incorporates ethionine into protein), a more recent claim is made (28) for such activation.

Although enzymes which catalyze specifically the activation of L-methionine (25, 30), L-tryptophan (31), L-tyrosine (32, 33, 34, 35), L-alanine (36, 37), L-threonine (33, 35, 38), L-serine (39), D-alanine (29), L-valine (25), L-isoleucine (25), L-leucine (25), and L-arginine (40) have been isolated in more-or-less highly purified form, many enzymes remain whose presence has been established, but which require further purification. Of particular interest is the question of whether cysteine and cystine are activated by the same enzyme, and whether they are incorporated into protein as cysteine or as two distinct amino acids.

Mechanism of action of activating enzymes.—The detailed mechanism of action of the activating enzymes may differ somewhat from enzyme to enzyme. Webster (37) has found that highly purified alanine-activating enzyme from pig liver is inhibited by sulfhydryl compounds and is unaffected by *p*-chloromercuribenzoate, in contrast, for example, to the tryptophan enzyme (31) or the tyrosine enzyme (32) from pig pancreas, which appear to depend on free sulfhydryl groups. Moreover, the tyrosine enzymes from rat liver (33), guinea pig liver (35), and hog pancreas (32) are all activated by potassium ion, in contrast to other activating enzymes.

The proof of the enzymic formation of amino acyl adenylates was incomplete until tryptophanyl AMP was isolated, substrate amounts of highly purified tryptophan-activating enzyme being used (41 to 44); further proof comes from the isolation of seryl AMP by Webster & Davie (39). An unidentified compound was also found (45) that was apparently an artifact, although its formation occurs to a greater extent during the isolation of

enzymically produced seryl AMP than during the isolation of the synthetic compound (39). The carbon atoms of serine become incorporated into the compound, but not the elements of ATP.

The finding of Boman & Svensson (46) that the formation of methionyl sRNA by yeast methionine-activating enzyme was greatly stimulated by the addition of partially purified *E. coli* arginine-activating enzyme was explained by the hypothesis that the *E. coli* enzyme preparation also contains an enzyme which reactivates partially inactivated activating enzymes. Although additional evidence is given which supports this hypothesis, the results must be interpreted with caution since the enzyme preparations used were not in a highly purified state. In addition, the enzyme was assayed solely by amino acyl sRNA formation rather than by PP^{32} exchange or hydroxamate formation, thus making it difficult to dissociate possible effects of the stimulatory factor on the sRNA from effects on the activating enzyme itself.

Methodology.—Von der Decken (47) has devised a rapid scanning procedure for amino acid-activating enzymes which is based on the paper chromatographic separation of the labeled ATP formed by PP^{32} exchange, and Bucovaz & Davis (48) have used Norite or Sephadex treatment to lower the non-amino acid-dependent PP^{32} exchange rates of tissue extracts, thereby increasing the sensitivity of the method for detecting the presence of activating enzymes.

THE FORMATION OF AMINO ACYL sRNA

The synthesis of amino acyl derivatives of sRNA has been studied in considerable detail by Berg *et al.* (49), and the general characteristics of the system confirm previously published results of many investigators (7). ATP could not be successfully replaced by UTP, GTP, CTP, deoxy-ATP, or ADP, and likewise no other RNA or synthetic polynucleotide which was tried could replace sRNA. As was found previously by Leahy *et al.* (50), the formation of the amino acyl derivatives of sRNA is a reversible reaction and, from the equilibrium constants [(about 0.3 to 0.4); also see Lipmann *et al.* (38)], it is evident that the free energy of hydrolysis of the amino acyl sRNA is of the same order of magnitude as that of the pyrophosphate group of ATP. This finding is not a surprising one, in view of the observed free energy of hydrolysis of amino acid esters (52).

Specificity of the transfer of the amino acyl group from enzyme-bound amino acyl AMP to amino acyl sRNA.—As was pointed out previously, the specificity of amino acid-activating enzymes for amino acids is not absolute, and is probably insufficient to account for the apparent lack of "mistakes" found in amino acid-sequence studies, particularly in the case of RNase, in which virtually every step in the determination of the sequence has been performed quantitatively [e.g., see (53)]. We can thus expect that the identity of the amino acid will not have become completely subordinated to that of the activating enzyme to which it is bound, and that, in one or more succeeding steps, the amino acid itself will have to play some role in determining speci-

ficity. This expectation is borne out by studies of the step succeeding the activation step, that is, the transfer of the amino acid from the enzyme-amino acyl adenylate complex to sRNA. With the *E. coli* isoleucine-activating enzyme which also catalyzes the formation of valyl adenylate, Bergmann *et al.* (25) have found that only the isoleucyl residue is transferred to the sRNA. Hence, given a choice of enzyme-amino acyl AMP complexes, the sRNA must be able to "recognize" the proper combination of catalytic protein and amino acid. In relation to the known amino acid specificity of RNA chains, the results of this experiment mean that the isoleucine enzyme can transfer the isoleucyl residue to isoleucine-specific sRNA but cannot transfer the valyl residue, nor can it transfer the valyl residue to valine-specific sRNA, which the valine enzyme can do. The idea that recognition of the combination occurs receives further support from results which show that, although tryptophan-activating enzyme can bind a number of amino acyl adenylates (catalyzing the formation of ATP) (54, 55), tryptophan is the only amino acid which can be transferred to sRNA (56). The apparent reduced specificity of the back reaction catalyzed by this enzyme, as compared with the forward reaction, is a matter of K_m values; it was found (57) that, when very high concentrations of amino acid were used, the specificity in the forward reaction was also reduced.

Heterogeneity of sRNA and methods of fractionation.—It is obvious that the high degree of uniqueness imparted to the enzyme-bound amino acyl sRNA by the two steps involved in its formation would be wasted, were not the sRNA to which the amino acid is transferred comprised of sites specific for individual amino acids. The evidence for such sites has come from studies on the destruction, by periodate, of all acceptor sites but the ones occupied by amino acids (58, 59), and from saturation experiments (25) which showed that the sRNA acceptor sites for different amino acids are independent of one another. Evidence that the specific sites are associated with individual sRNA molecules has been based on fractionation of sRNA or amino acyl sRNA by ion exchange or partition chromatography (60, 66), electrophoresis (38), or countercurrent distribution (61), on fractionation based either on the specific functional groups possessed by some amino acids (62), or on the oxidation by periodate of the acceptor sites not occupied by amino acids (63).

These studies have led to only partial separations, and attempts to isolate individual sRNA chains by new techniques and by refinements of older techniques have continued. Smith *et al.* (64; and see 65) have employed a procedure which depends on the chromatography of sRNA on the anion exchanger Cato-2, an ungelatinized tertiary amino alkyl ether of starch. A partial separation of specific sRNA chains was accomplished and, in addition, the ability of the procedure to distinguish between sRNA and microsomal RNA permitted the detection of sRNA in washed microsomal preparations. A partial separation of leucine-specific and valine-specific sRNA was achieved by Ofengand *et al.* (66), using Ecteola-cellulose and sRNA charged with these

amino acids. By a similar technique, but with DEAE-cellulose substituted, Nishiyama *et al.* (67) have partially purified leucyl sRNA.

Fractionation by counter-current distribution has been continued by Doctor *et al.* (68). Four hundred transfers of yeast sRNA gave a tenfold purification of valine-specific and tyrosine-specific sRNA, but it was evident that no individual RNA free of acceptor activity toward all other amino acids was obtained. Preliminary investigation of base composition, optical rotation, and spectra indicated that significant structural differences do exist among the different RNA fractions. This view is reinforced by the ease of separation of valine-specific sRNA from tyrosine-specific sRNA by only six transfers (69), and by an extension (70) of these studies which has led to the isolation of alanine-, valine-, and tyrosine-specific RNA chains in apparently highly purified form. Analyses of base composition and chromatography of RNase digests indicate appreciable differences in structure.

The solvent system used in the counter-current distribution system (68) can also be adapted to partition chromatography on silicic acid (71). While no single RNA was obtained, in the case of tyrosine, tryptophan, histidine, valine, and alanine, each of these was completely separated from the other four, with a tenfold purification noted for the tyrosine-specific and the alanine-specific RNA. Another system which offers promise is chromatography on hydroxyapatite (72) which has been used by Hartman & Coy (73) to fractionate *E. coli* sRNA. The valine-specific RNA underwent an elevenfold increase in specific activity and was completely separated from the phenylalanine activity. In contrast to the results of Doctor *et al.* (68) and Holley *et al.* (70) on fractions obtained from yeast sRNA, the *E. coli* alanine and valine fractions were found to possess similar base ratios. Cantoni (74) has been able to achieve some fractionation of rabbit liver sRNA by the simple expedient of adding spermine.

Methods of fractionation based on the dialdehyde formed when periodate reacts specifically with the free (i.e., unoccupied by an amino acid) vicinal hydroxyls of the terminal ribose of sRNA (58) have been extended. Portatius *et al.* (75) have treated valine-charged sRNA from yeast with periodate and permitted the RNA-dialdehyde thus formed to react with the water-soluble polymer, polyacrylic acid hydrazide. The resulting compound was then precipitated by reaction with *n*-butyraldehyde, leaving valyl sRNA in the supernatant fluid in good yield with a tenfold increase in specific activity and a calculated purity of about 28 per cent. A procedure similar in principle was used by Saponara & Bock (76) who, after periodate treatment, reacted the sRNA-dialdehyde with a phenylhydrazine resin. After the resin was filtered off, a valyl RNA of increased specific activity was left free in solution.

In the very effective procedure reported by Stephenson & Zamecnik (77), the dialdehydes formed after periodate treatment are reacted with 2-hydroxy, 3-naphthoic acid hydrazide—the resulting hydrazone then being linked to a dye (tetrazotized *o*-dianisidine). The soluble mixture is then chromatographed

on DEAE-Sephadex which carries out an effective separation of valyl-RNA and dye-RNA. Based on a molecular weight of 25,000 the valyl-sRNA was calculated to be 90 per cent pure.

The experimental demonstration of the existence of individual sRNA chains, each of which is specific for an individual amino acid, is in accord with the specificity requirements of protein synthesis and with the adaptor hypothesis [Crick (24)]. Some modification of the latter idea may be necessary, however, because of the discovery, in the same tissue, of different kinds of sRNA specific for the same amino acid. The evidence for this sub-heterogeneity is as follows: With *E. coli* sRNA, Berg *et al.* (49) found that of the total methionine sites available, as measured with the *E. coli* methionine-activating enzyme, 60 per cent of these were inert to the yeast enzyme. The study of the sites which survived periodate oxidation after use of each of these enzymes confirmed these findings. Thus, these results constitute evidence, not only of a species difference between two methionine-activating enzymes, but of the existence of two different kinds of methionine acceptor sites on the sRNA from a single organism. Similar conclusions can be drawn from the results obtained by Allen *et al.* (78) with a different system. The possible generality of this conclusion receives support from the results of Doctor *et al.* (68) who found that after counter-current distribution of yeast sRNA the leucine-specific and threonine-specific peaks were heterogeneous. However, in the experiments of Benzer & Weisblum (79), in which *E. coli* and rabbit liver extracts were tested with *E. coli* sRNA and arginine, and yeast and rabbit liver extracts were tested with yeast sRNA and tyrosine, no evidence could be found for heterogeneity of either the arginine-specific or the tyrosine-specific RNA acceptor sites.

Attempts to explain the findings on the sub-heterogeneity of sRNA in the light of present concepts of its role in protein synthesis lead to a number of hypotheses: (a) some sites on the template may not permit the attachment of an RNA chain of a particular structure because of the presence, for example, of a nearby unfavorable sequence of bases; (b) in the intact cell, the two kinds of methionine-specific sRNA are localized in two different subcellular structures, and are involved in two structurally distinct systems of protein synthesis—for example, good evidence exists for nuclear protein synthesis (13), and a difference in structure between nuclear and cytoplasmic sRNA has been found (80); (c) more than one methionine-activating enzyme exists in the cytoplasm, with each enzyme requiring a specific sRNA; (d) the RNA-protein code is a degenerate one, so that more than one kind of sRNA might be needed to recognize each of the different code words on the template; (e) sRNA has a dual function; and (f) the heterogeneity is an artifact.

Species specificity.—Further evidence of species differences between activating enzymes comes from the work of Rendi & Ochoa (81), who have demonstrated that the leucine-activating enzyme in *E. coli* can transfer leucine only to homologous sRNA. It has also been shown [Webster *et al.* (80)] that

pig liver activating enzyme will transfer amino acid to pig liver sRNA and pig muscle sRNA, but not to sRNA from calf liver, yeast, peas, or pig liver nuclei. In a systematic investigation of this problem, however, Benzer & Weisblum (79) have made the important observation that the interchangeability of activating enzymes and sRNA from different organisms depends not only on the organisms involved but on the particular amino acid used. Thus, generalizations as to species specificity of the different components involved in protein synthesis can validly be made only if the tests are done with a multiplicity of amino acids.

Structure of sRNA.—A recent review on this subject includes new studies by Berg and co-workers (7) on the nucleotide sequence. In addition, it is only necessary to note that the apparent independence of the amino acid-acceptor activity of sRNA on its secondary structure (82) has been confirmed (83), that a helical model of sRNA has been constructed which is based on a two-stranded structure as similar as possible to DNA (84), that sRNA in the intact cell is considered to exist as a nucleic acid-protein complex (85), and that the presence of the methylated bases in sRNA may be important for its activity (86).

THE TRANSFER OF THE AMINO ACID FROM AMINO ACYL sRNA TO RIBOSOMAL PROTEIN

Requirements for the transfer.—In contrast to the reactions responsible for the formation of amino acyl sRNA, very little is known about the detailed chemistry of the subsequent steps leading to the synthesis of the completed protein molecule. Earlier observations along these lines (7, 20) may be summarized as follows: The major requirements for the transfer of an amino acid from amino acyl sRNA to ribosomal protein consist of ATP, GTP (20), an enzyme (20, 87, 88) which has been partially purified from the high speed supernatant fraction (89), reduced glutathione in certain cases (90 to 94), amino acyl sRNA, and washed ribosomes. The amino acyl sRNA appears to be transferred to the ribosomes as such (20, 90, 95, 96, 97), and the sRNA becomes attached by a linkage resistant to 8 *M* urea and ethylenediaminetetraacetic acid (98), although these observations cannot be construed to provide sufficient evidence for covalent bonding.

Hoagland & Comly (97) studied the incorporation of amino acyl sRNA into the ribonucleoprotein of ascites tumor microsomes and found that the amino acid was incorporated at the same rate as the RNA, but that the extent of incorporation of RNA reached a plateau much sooner than did the amino acid. These observations led to the conclusion that essentially all of the sRNA molecules accompany the attached amino acids into the particles during the transfer reaction, and that there are a limited number of sRNA sites on the ribosomes which are rapidly filled with amino acyl sRNA, the amino acid being transferred to a growing peptide chain and the sRNA shuttling back to the soluble fraction to pick up more amino acid (see later dis-

cussion). The reaching of a plateau in amino acid incorporation may mean either that the system becomes inactivated or that all the amino acid sites are filled and that as much finished protein is leaving the particles as there are new protein chains being synthesized on the particles. Experiments to be described later would favor the latter explanation. The further observation that the rate of incorporation into rat-liver microsomal RNA is the same for sRNA with or without attached amino acids has been shown to be true for rat-liver ribosomes as well [Bloemendal *et al.* (103)]. Although sRNA incorporation in the latter system is not stimulated by amino acids, or by a soluble fraction containing activating enzymes, the possibility is not ruled out (97, 103) that the sRNA was first acylated to form amino acyl sRNA, and then incorporated. The dependence of the sRNA transfer on GTP addition speaks against this, however, and may indicate that the GTP functions specifically in the formation of the peptide chain, and not in the binding of amino acyl sRNA. Studies [Bosch & Bloemendal (99)] with the antibiotic, puromycin, which also support the idea that free sRNA can be bound to microsomal RNA, are discussed in the next section.

Mechanism of action of puromycin.—Yarmolinsky & de la Haba (100) have shown that puromycin inhibits the transfer of amino acid from amino acyl sRNA to ribosomal protein in a rat-liver system, and this observation has been confirmed (89, 99). The results of Nathans & Lipmann (101) with *E. coli* show that puromycin promotes the hydrolysis of amino acyl sRNA (or of some amino acyl compound in equilibrium with it), and that the puromycin-catalyzed hydrolysis is dependent upon the presence of ribosomes, the transfer enzyme, GTP, phosphoenolpyruvate, and pyruvate kinase; however, the site of action of the antibiotic appears to be directly on the ribosome. The action of puromycin in promoting an attack by water is reminiscent of the effect of dinitrophenol on oxidative phosphorylation, where phosphate transfer degenerates to hydrolysis. Whether puromycin is bound to the ribosome, or whether it acts as a competitive analogue of sRNA (99), is not known. Although puromycin completely inhibited the transfer of the amino acyl moiety of amino acyl sRNA to microsomal protein, the enzymatic transfer of free sRNA to ribosomal RNA was unaffected [Bosch & Bloemendal (99)]. Thus, the transfer of sRNA to ribosomes may take place without an attached amino acid although, as pointed out previously, such an interpretation is complicated by the possibility that, as in the results of Hoagland & Comly (97), the sRNA might first have become acylated by endogenous amino acid, then transferred to the ribosome, with the subsequent hydrolytic removal of the amino acid.

Studies on the transfer enzyme.—A partially purified transfer enzyme has been obtained by Nathans & Lipmann (101) from *E. coli*. The preparation is free of amino acid-activating activity, and the transfer activity is found to emerge from a DEAE-cellulose column at the same position, no matter which labeled amino acid is used in the assay. These results indicate that either only

one such enzyme exists, in accord with the results of Grossi & Moldave (102), or that the procedure does not resolve a possible multiplicity of enzymes. The transfer of the amino acid requires the labeled amino acyl sRNA, the other (unlabeled) amino acyl sRNA compounds, well-washed ribosomes, GTP, phosphoenolpyruvate and its kinase; the system also shows a partial requirement for reduced glutathione. ATP is not required, and in a rat-liver system [Takanami & Okamoto (88)] phosphoenolpyruvate and pyruvate kinase may be omitted as well. The sRNA recovered after incubation (101) appeared to be intact; its leucine-acceptor activity was equal to that of the control, and it failed to accept AMP as an end group. These observations, along with those of Scott [see (97)], who studied the turnover of sRNA and of its terminal adenosyl residue in intact ascites tumor cells, suggest that the sRNA remains intact during its function as a shuttle between ribosome and soluble fraction, and that its role may be compared with that of a coenzyme such as coenzyme A. In possible disagreement with this view is the observation of Bloemendal *et al.* (103) that the labeled terminal adenine of sRNA appears in an internal position of microsomal RNA, but it has not been shown that this reaction is related to the amino acyl transfer reaction.

Bishop & Schweet (104) have purified the transfer enzyme from reticulocytes by salting out, by chromatography on Sephadex and DEAE-cellulose, and by adsorption on calcium phosphate gel, and have found that the function of added glutathione or other sulfhydryl compounds is to activate the transfer enzyme. Full activity can be obtained without glutathione addition if the enzyme is isolated in the presence of EDTA. In contrast, Webster (105), using pig-liver transfer enzyme, has found the requirement for glutathione to be specific, and Takanami (106) has found it to inhibit the purified rat-liver enzyme. Further evidence of the sulfhydryl nature of the reticulocyte enzyme comes from experiments (104) with *p*-chloromercuribenzoate, *N*-ethylmaleimide, and silver nitrate.

Von der Decken & Hultin (94) considered that their results suggested the existence of transfer enzymes specific for individual amino acids, and Bishop & Schweet (51) have obtained results in accord with this view. Chromatography on DEAE-cellulose gave four fractions, each of which showed only slight leucine-C¹⁴ transfer activity, but when two of these were combined, a tenfold increase over the sum of the separate activities was observed. In addition, the various fractions showed a marked differential specificity toward amino acid transfer which was interpreted as an indication for a separate transfer enzyme for each amino acid. This differential amino acid specificity is difficult to understand, however, when it is considered that if normal proteins are being formed, the decrease in, or the loss of, the ability of the system to incorporate a particular amino acid should be reflected in an almost equal decrease in the ability to incorporate all amino acids. The possibility that the results can be explained by the presence of components in the various fractions other than, or in addition to, the transfer enzyme, rather than by a

multiplicity of amino acid specific transfer enzymes, cannot be assessed at present.

Species specificity.—Studies of species specificity in the transfer step must take into account the biological sources of the amino acyl sRNA, of the transfer enzyme, and of the ribosome, and the results of the studies may depend upon the particular amino acid used (see discussion on amino acyl sRNA formation). The questions of whether additional enzymes are involved, or whether so-called "messenger" RNA (see later discussion) possesses species specificity, are under investigation in many laboratories, but definitive answers are not yet available. Complete studies in which the sources of each of the three known components of the transfer reaction were varied systematically with respect to one another, and in which purified components have been used, are not available. However, it is possible to draw some tentative conclusions from results which have been reported.

Earlier observations on the species specificity of the transfer step have made use of the pH 5 enzymes (which contain amino acid-activating enzymes and sRNA) or amino acyl sRNA from one species and ribosomes or microsomes plus pH 5 supernatant fluid (containing transfer enzymes) from another. It appears that amino acyl sRNA from yeast or *E. coli* is equivalent to mammalian amino acyl sRNA in its ability to donate the amino acid to mammalian ribosomes (20, 38, 89), and likewise, pea seedling ribosomes [Mans & Novelli (107)] show no species discrimination in a test with rat-liver pH 5 fraction. The results also indicate that there is no species discrimination between sRNA and transfer enzyme. In contrast, the pH 5 enzymes from a Rous sarcoma virus-induced tumor were inactive in the rat-liver microsomal system [Wagle *et al.* (108)], thus suggesting discrimination, in this case, against heterologous sRNA by either transfer enzyme and ribosome, or both. In a study in which the source of the amino acyl sRNA and of the ribosomes was held constant (both from rat liver) and the source of the transfer enzyme was varied, Nathans & Lipmann (89) found that the transfer enzymes from various animals could substitute for the rat-liver enzyme, thus substantiating the lack of species specificity between sRNA and transfer enzyme and, likewise, demonstrating a lack of such specificity between transfer enzyme and ribosome. However, when the source of the amino acyl sRNA (*E. coli*) was kept constant but the sources of the transfer enzyme and of the ribosomes were varied (101), it was found that: (a) the *E. coli* enzyme was ineffective in combination with rat-liver ribosomes; (b) the rat liver enzyme would not transfer the amino acid to *E. coli* ribosomes. Similar results were obtained by Rendi & Ochoa (81). Result (a) indicates a discrimination by rat-liver ribosomes against either the transfer enzyme or the sRNA from *E. coli*. Inasmuch as *E. coli* sRNA is effective with mammalian ribosomes (see above and 20, 38, 81, 89), the species specificity exists between the ribosome and the transfer enzyme. The same results (20, 38, 81, 89) indicate that *E. coli* sRNA is effective with mammalian transfer enzyme; this indicates that result (b) may be

interpreted in similar fashion to result (a). However, generalizations from these studies (89, 101) to the effect that transfer enzyme-ribosome species specificity is a function only of the proximity of the biological relationship of the species may be premature; (a) rat liver and *E. coli* high-speed supernatant fluids rather than purified transfer enzymes were used—hence, while positive results obtained under such conditions can be controlled somewhat, negative experiments might be the result of mere technical difficulties; (b) a different source of sRNA was used in each study—e.g., there is no experiment (89) in which a combination of rat-liver amino acyl sRNA, rat-liver ribosomes, and *E. coli* transfer enzyme is used; (c) the tests were conducted only with leucyl sRNA.

The results of the various types of specificity experiments (species and otherwise) which have been carried out on sRNA indicate that this molecule is called upon to recognize (and be recognized by) a specific amino acid, a specific activating enzyme, possibly an amino acid-specific transfer enzyme (108), and, presumably, a specific site on the template, besides providing a site of attachment for the amino acid. In addition, it must, in certain cases, distinguish between the same amino acid-activating enzyme from different species, and perhaps a species specificity exists for the transfer enzymes as well (108). Moreover, the number of template recognition sites could conceivably exceed one, if the RNA-protein code is degenerate (see later discussion). It would seem that the sRNA molecule is none too large or none too complex for this multiplicity of functions.

The interpretation of heterologous experiments is made clearer by studying the effects on specific proteins: does their synthesis continue and, if so, is there any change in their structure? The suggestion has been made by Benzer & Weisblum (79) that, during species variation, a likely site for a mutational change might be either the amino acid "recognition" site or the template recognition site on the sRNA, or both. Assuming that appropriate nucleotide sequences on the RNA template recognize not amino acids but only specific nucleotide sequences on the sRNA [adaptor hypothesis (24, 117)], the use of heterologous sRNA could lead to one or more of several results. (a) If the RNA-protein "code" is universal, and provided that the synthesis of the specific protein is not totally inhibited by components of the system (e.g., transfer enzyme) whose activity might depend on nucleotide sequences in sections of the sRNA which are not concerned with this code, a normal protein should be formed. (b) If the code in the two species differs for a very few amino acids, and again assuming no block in synthesis because of trivial reasons, amino acid replacements should occur provided that the other code makes "sense" to the heterologous template. (c) If the code is very different in the two organisms, the specific protein would not be formed, and the synthesis of proteins or polypeptides would either stop or proteins of jumbled sequences would be formed. The latter situation might not be recognized

unless the synthesis of a specific protein were being studied. (d) Should the specific protein synthesized fail to correspond absolutely to the source of the ribosome, but correspond rather to the source of the sRNA preparation or of the soluble fraction of the cell, then either our current ideas about the role of sRNA (including the unproven adaptor hypothesis) are incorrect, or some other amino acid sequence-specifying substance is present in the sRNA preparation or in the soluble fraction.

The first attempt to come to grips with the problem of the synthesis of specific proteins by heterologous systems was made by Campbell *et al.* (109, 110) who found, using immunological techniques, that rat microsomes synthesized rat serum albumin exclusively, whether rat or duck soluble fraction was used. In this and the other experiments on specific proteins to be discussed in this section, amino acid incorporation is taken to mean protein synthesis, with the realization that net synthesis has not, in fact, been demonstrated. When the ribosomes and the supernatant fluids of rabbit and mouse reticulocytes were interchanged, only that type of hemoglobin was formed which corresponded to the source of the ribosomes [Bishop *et al.* (111)]. To meet the objection that the sRNA which is active is not the added material but that bound to the ribosomes (112), C^{14} -leucyl sRNA from guinea pig liver was substituted for C^{14} -leucine and sRNA, and was tested with rabbit reticulocyte ribosomes; again, rabbit hemoglobin was formed (111). In an experiment with C^{14} -amino acyl RNA from *E. coli* and mammalian reticulocyte ribosomes, von Ehrenstein & Lipmann (113) found that mammalian hemoglobin was formed. Further experiments by Bishop *et al.* (111), in which the isolated labeled hemoglobin was separated into its α and β chains, subjected to partial hydrolysis, and fingerprinted, showed that the relative labeling of the peptides was the same whether the hemoglobin had been labeled by intact rabbit reticulocytes and free C^{14} -leucine, by guinea pig C^{14} -leucyl sRNA and rabbit ribosomes, or by a homologous cell-free rabbit system. These results provide strong and definitive evidence of the cell-free synthesis of hemoglobin, and of the synthesis of rabbit hemoglobin by the heterologous system.

The pre-eminent role of the ribosomes in specifying protein structure, as seen above, has been sharply challenged by the results of Kruh *et al.* (114), who found that when either rabbit-reticulocyte microsomes and guinea pig pH 5 fraction or the alternate combination of components was used, both rabbit and the guinea pig hemoglobin were formed. Furthermore, the proportion of the two hemoglobins synthesized depended upon the proportion of microsomes and pH 5 enzymes from the two species. Such divided specificity has also been found independently by Lamfrom (115) who, in a system composed of pH 5 fraction, pH 5 supernatant fluid, and microsomes, tested these three fractions from sheep and rabbit reticulocytes against one another in all possible combinations. The specificity factor seemed to be in the microsome

and the pH 5 supernatant fluid. Indications that the pH 5 fraction might also exert some effect are difficult to evaluate; the author points out that this fraction may be contaminated with microsomes.

The difference between the results of Bishop *et al.* (111) which show complete microsomal control of specificity (also see some of the systems used by Kruh *et al.*), and those of Lamfrom (115) and of Kruh *et al.* (114) which show divided specificity, is difficult to understand. The explanation could lie in the mechanism by which the finished hemoglobin molecule is released from the ribosome; under given experimental conditions, for example, although two types of hemoglobin might have been synthesized, only the homologous kind might be released. (See later section on Nascent Proteins.)

The interpretation of species-specificity experiments depends to a great extent on the purity of the tested fractions. It is possible, for example, to interpret in a converse manner results which ostensibly indicate that the specificity lies with the ribosomes and not with the sRNA, particularly since the experiments discussed above [Bishop *et al.* (111); von Ehrenstein and Lipmann (113)], which were designed to eliminate the participation of endogenous sRNA, may not do so. For example, the possibility of an amino acyl exchange occurring between endogenous sRNA bound to the ribosome and added amino acyl sRNA has not been tested. Such an exchange might occur directly (assuming the existence of an appropriate enzyme) or could take place via amino acyl AMP in the presence of a catalytic amount of AMP and traces of the appropriate activating enzyme. Thus, heterologous experiments yielding a specific protein which corresponds to the source of the ribosome can be explained by the failure of the added heterologous sRNA to function directly, and experiments yielding both types of proteins can be explained by both the endogenous and the heterologous sRNA being active, possibly because of slightly different experimental conditions. An alternative explanation of the experiments of Lamfrom (115) and Kruh *et al.* (114) is that the specificity is determined solely by the ribosomes, but that these particles contaminate the pH 5 preparation or the pH 5 supernatant fluid. Control experiments [Lamfrom (115)] point to such contamination, but it is unlikely that the contamination can be large enough to account for the results.

It is, of course, possible that some as-yet-unidentified substance exists which can specify the amino acid sequence of a protein, and which can be present both in the ribosome and in the soluble fraction. Recent evidence, obtained from microorganisms, of the existence of such a substance, sometimes termed "messenger" RNA, will be presented in a later section.

Is sRNA an obligatory intermediate in protein synthesis?—The large body of evidence, based on amino acid-incorporation studies in microsomal and ribosomal systems, that sRNA acts as an intermediate in protein synthesis [consult (7, 19, 20, 22) and the previous discussion on transfer enzymes], is reinforced by the isolation of amino acyl sRNA compounds from intact cells (95, 116, 118), and by their implication as substrates for the net synthesis of

protein by pea seedling ribosomes (119). More recently, direct evidence has been obtained of the transfer of amino acids from labeled amino acyl sRNA compounds to specific proteins. In the course of the studies by Campbell and his group on the synthesis of serum albumin by rat-liver microsomes (110, 120), and by deoxycholate-prepared ribosomes (121) [also see Korner (122)], it was found (123) that purified C^{14} -leucyl sRNA would serve as a precursor for the synthesis of C^{14} -labeled serum albumin in the rat-liver microsomal system. Results obtained independently by Hirokawa *et al.* (124) are in agreement with these findings. Analysis of the partial hydrolysate of the C^{14} -albumin [Campbell *et al.* (125)] by paper electrophoresis indicated that the C^{14} -leucine had become incorporated into many peptides, but when the relative density of labeling of these peptides was compared with that of *in vivo*-labeled albumin, some differences were obtained (see also 126). Von Ehrenstein & Lipmann (113), using rabbit reticulocytes, have been able to demonstrate a 66 per cent transfer of leucine- C^{14} from purified leucyl sRNA to hemoglobin. The isolated and purified hemoglobin was partially hydrolyzed, and the labeled peptides, when subjected to finger-printing, coincided with peptides obtained in similar fashion from authentic hemoglobin.

While these studies offer strong evidence that amino acyl sRNA can act as an intermediate in the synthesis of protein, they do not prove conclusively that it is an obligatory intermediate; it could, for example, be the product of a side reaction and be in equilibrium with some component, X, in the main path of protein synthesis. Such a possibility would be difficult to eliminate in kinetic experiments if the rate of equilibration with X were more rapid than the rate of turnover of X itself. However, if it be tentatively assumed that this is not the case, then the experiments of Moldave (127), in which the rates of incorporation of a labeled amino acid into sRNA and into microsomal protein of rat liver were measured, support the obligatory role of sRNA. Allen & Schweet (112), employing the rabbit reticulocyte system, labeled the sRNA of the soluble fraction by preincubating it with leucine- C^{14} , after which carrier leucine and ribosomes were added simultaneously. The total C^{14} found in the protein corresponded to the proportion of C^{14} in the leucyl sRNA, thus indicating that all the protein radioactivity had passed through the leucyl sRNA. However, unless the previously discussed assumption is made, proof for the obligatory role of sRNA is not conclusive. In contrast to these observations, Raacke (128), studying the net synthesis of protein in a pea seedling system, could find no requirement for sRNA; how much was bound to the ribosomes was unknown. Furthermore, Rychlik (129), using a rat liver microsomal system, found that concentrations of 6-azauridine-5'-monophosphate which caused a 98 to 99 per cent inhibition of amino acyl sRNA formation, inhibited amino acid incorporation into protein by only about 50 per cent. This evidence would be strong, indeed, had the possibility been eliminated that sRNA which may be bound to the ribosomes (64, 112) was not affected by the inhibitor.

The findings of Bates & Lipmann (130, 131) that sRNA is involved in the synthesis of glutathione as a glutamylcysteinyl-sRNA intermediate are discounted in their entirety by Lane & Lipmann (132).

The role of GTP.—Beyond the fact that the action of GTP is localized somewhere in the pathway between amino acyl sRNA and the completed protein molecule, little is known of its mechanism of action. Two exceptions to the sharply defined specificity for GTP may be noted. One comes from the work of Rogers & Novelli (133) who, in their studies on the microbial synthesis of ornithine transcarbamylase, found a requirement for UTP and CTP as well as GTP. It is not known, however, whether these requirements are related to the usual GTP effect. The other exception is a ribosomal system derived from thymus nuclei, in which CTP can completely replace GTP [Wang (134)]—a finding not in accord with that of Frenster *et al.* (135) who demonstrated a specific GTP requirement. With pea seedling ribosomes and amino acyl sRNA as the substrate, Webster *et al.* have reported that GTP hydrolysis accompanies, and is dependent upon, the conversion of the amino acid to peptide linkage, provided that an enzyme which catalyzes an apparently unrelated hydrolysis of GTP is removed from the transfer enzyme (136, 137). It is also reported that amino acids can be transferred from ribosomal-bound polypeptide to sRNA with a concurrent formation of detectable amounts of GTP.

NASCENT PROTEINS ON THE RIBOSOME, AND THEIR RELEASE

If ribosomes are the site of the final steps in protein synthesis, then newly formed proteins should be found associated with these particles. Three general approaches have been used to gain information about these proteins: (a) attempts to distinguish between the products of ribosomal synthesis and the structural protein of the ribosome; (b) attempts to identify specific proteins associated with the ribosome; and (c) studies of the mechanism by which newly synthesized proteins are released from the ribosome.

Labeling experiments.—Indications that a portion of the protein on the ribosomes turns over rapidly, and that the nascent protein is transferred to the soluble fraction, first came from pulse-labeling experiments with intact reticulocytes [Rabinovitz *et al.* (138); Dintzis *et al.* (139)]. Inasmuch as the protein synthesized by reticulocytes is largely hemoglobin (140), these (138, 139) and other results [Wallace *et al.* (141)] suggested that in short time-interval experiments such as the above, most, if not all, of the radioactivity in the ribosomes is in the form of newly synthesized globin, hemoglobin, or immediate precursors thereof, and is not newly synthesized "structural" protein. Fractionation of the pulse-labeled ribosomes in an aqueous detergent-organic solvent partition system showed that neither the RNA nor the basic proteins were appreciably labeled, whereas a high proportion of the radioactivity was associated with a protein of very high specific activity, extractable with water from the solid interfacial layer and not bound to RNA. Since

virtually all the structural proteins of the ribosome (at least from *E. coli*) are basic in nature [Waller & Harris (142)], these results suggest that the rapidly synthesized proteins are not the structural proteins but are being made for export to other parts of the cell—conclusions which are in accord with those reached by Young *et al.* (166) from pulse experiments on yeast. Further, these proteins (141) are not covalently linked to RNA unless the linkage is very labile or is very short lived so that, at a given moment, few molecules will be so bound. At low Mg^{++} concentrations, the ribosomes dissociate into smaller subunits and release a small amount of protein of very high specific activity, probably the nascent protein.

Isolation of specific proteins.—A number of attempts have been made to detect the presence of specific proteins on the ribosome (as distinct from microsome). Perhaps the first reported case of enzymatic activity so associated was that of an RNase bound to a ribonucleoprotein particle isolated from tobacco leaves [Pirie (143)]. The presence of this enzyme was later detected in ribosomes of *E. coli* (144, 145, 146, 147), of pea seedlings (148), of guinea pig pancreas (149), of liver (150), and of rabbit spleen (151). In *E. coli*, the enzyme appears to be confined to the 30S component of the ribosome (147, 152). Other enzymatic activities found associated with ribosomes are DNase (145, 147), leucyl amino peptidase (146, 147), proline peptidase (147), acid phosphatase (147), and alkaline phosphatase (167)—all in *E. coli*, triose phosphate dehydrogenase in yeast (167), amylase in pancreas (153), β -galactosidase (154) and β -glucosidase (155) in yeast, as well as antigens in liver, spleen, and lymph nodes (151, 156). The identification of these enzyme activities has often, because of their latency, required promoting their release from the particle, and this has been accomplished by a variety of procedures, such as treatment with urea (144, 145, 147, 152), NaCl (147, 158), ribonuclease (151), antisera specific for the enzyme being studied (154), sonic vibrations (121), or Mg^{++} complexing reagents such as ethylenediaminetetraacetic acid (147), pyrophosphate, or ATP (153).

While adsorption from the soluble cytoplasm has not been eliminated as an explanation for the presence of these enzymes in the ribosome (except in the case of RNase which does not appear free in the cytoplasm of *E. coli*), evidence has been provided that, at least in the case of β -glucosidase, a ribosome-bound enzyme is actually a precursor of the cytoplasmic enzyme [Kihara *et al.* (155)]. Based on the finding (159) that incubation of yeast cells with *p*-fluorophenylalanine permits, for a time, linear growth and incorporation of amino acids into yeast proteins, but causes the synthesis of inactive enzyme, the authors (155) incubated yeast cells with this analogue, but for short time intervals (5 min). Under these conditions, the total level of β -glucosidase activity in the cells remained constant, but the ribosome-bound activity fell sharply. The addition of phenylalanine restored the ribosomal activity. These results suggested that β -glucosidase is synthesized in the ribosome, and it remained to be demonstrated that the soluble enzyme was

identical to the ribosomal enzyme. Strong evidence for this view was provided by experiments in which the two enzymes were compared with respect to their physical and catalytic properties.

Mechanism of the release of protein from the ribosome.—The release of the completed protein molecule from the ribosome may be under metabolic control. Earlier experiments pointed to an energy requirement for the release of labeled serum albumin from liver microsomes [Peters (160)], of labeled soluble protein from reticulocyte microsomes [Rabinovitz & Olson (161, 162)], of labeled protein from liver microsomes [Simkin (163)], and of labeled antibodies from spleen microsomes (163) where the release was shown to require the soluble fraction of the cell in addition to ATP and Mg^{++} , as was also the case with reticulocyte ribosomes [Morris & Schweet (164)]. The release of protein from reticulocyte ribosomes was not caused by the degradation of the particles, since the ribosomes retained their 80S state after incubation. Evidence that the soluble protein released was hemoglobin came from its solubility behavior in ammonium sulfate and the identity of its chromatographic behavior with that of authentic hemoglobin.

The release of protein from the ribosomes of reticulocytes [Morris & Schweet (164)] and of ascites tumor cells [Rabinovitz & Fisher (157)] is stimulated by puromycin. If it were assumed that, before its normal release, the completed protein molecule is still bound to an sRNA chain (which had transferred the terminal amino acid to the protein), and that the sRNA chain is still linked to the ribosome, then the stimulation of the release of protein by puromycin might be explained by the assumption that cleavage of the protein-sRNA bond is promoted by puromycin, just as this substance seems able to effect the hydrolysis of ribosomal-bound amino acyl sRNA. Whether this hypothesis is also in accord with both the requirement for the soluble fraction (163, 164) mentioned above, and the observation [Hoagland & Comly (97)] that the addition of sRNA to the medium seems to displace labeled sRNA from the ribosome, is a matter for further investigation. However, the stimulation by the soluble fraction might also be explained by its possible content of a "release enzyme" which Webster (137) has obtained from extracts of pea-seedling ribosomes, but which is distinct from the transfer enzyme. Of a large number of substances and conditions tested in a rat-liver microsomal (not ribosomal) system, only NAD (137) stimulated total protein release and an increased K^+ concentration led to a seventeenfold increase in the amount of serum albumin released [Lingrel *et al.* (165)].

In contrast to most of these results, von Ehrenstein & Lipmann (113) have not found a requirement for ATP or soluble enzymes (other than those present on the washed ribosomes) for the release of hemoglobin from reticulocyte ribosomes. However, Lamfrom (115) has found a "releasing factor" in the supernatant fraction of reticulocytes of all species tested, and the evidence obtained from incubation experiments of *in vivo*-labeled microsomes or ribosomes from different species, has prompted Webster (137) to suggest that the

"release enzyme" may be active in some systems, but not in the rat-liver microsome system.

SPECIFICATION OF THE AMINO ACID SEQUENCE OF PROTEINS

The observations that enucleated halves of a green alga, *Acetabularia*, are capable of growth (168) and of the synthesis of total and specific proteins (169, 170) seemed to rule out a direct role for DNA in protein synthesis. This view was reinforced by the general failure to find DNA in the cytoplasm (169), and it was later assumed that the genetic information carried by DNA, which specifies the amino acid sequence of a protein, is permanently transferred to the cytoplasm and is present there as the base sequence of ribosomal RNA. Studies of the past few years on cell-free systems also offer strong evidence of the synthesis of protein in the cytoplasm and therefore, in the following discussions, the assumption is made that DNA does not play a direct role as a template for protein synthesis. [For further discussion of this question, consult (7, 169).]

*The evidence for a new rapidly synthesized RNA.*⁴—According to current ideas on the role of RNA in protein synthesis, prior to the synthesis of a protein which is entirely new to the cell, the formation of a new RNA "template" should occur, and this should be manifested by a net synthesis or increased turnover rate of RNA. The formation of a new protein in response to a change in the genotype of the cell has permitted such studies on cellular RNA. One such change results from phage infection [Lwoff (171)] of *E. coli*; after infection, no net synthesis of RNA can be detected (172 to 175), but Hershey (176, 177) and Volkin *et al.* (175, 178, 179) have discovered that an immediate rapid turnover of RNA takes place. Other results which suggest the rapid synthesis of some cytoplasmic product of gene action were provided by the experiments of Pardee *et al.* (180), which showed that when the β -galactosidase gene is transferred to an *E. coli* strain that is normally unable to synthesize this enzyme, the synthesis starts almost immediately. Moreover, P^{32} decay experiments show that the decrease in the rate of synthesis of the enzyme follows closely the rate of DNA- P^{32} decay [Riley *et al.* (181)], thus indicating a short half-life for the cytoplasmic intermediate.

One hypothesis common to the above experiments is that DNA is responsible for the synthesis of a special kind of RNA, which is rapidly synthesized, metabolically unstable, and which is responsible for the synthesis of protein. Further, because the cell presumably would not have previous "knowledge" of the structure of the new proteins, the special RNA is assumed to carry in its base sequence the information requisite for specifying the amino acid

⁴ For the purposes of the following discussion, when the base compositions or base ratios of nucleic acids are compared, no differentiation is made between thymine and uracil or between cytosine and hydroxymethylcytosine, and minor components such as 5-ribosyluracil or the methylated adenines and guanines, etc., are ignored.

sequence of a protein; that is to say, the RNA plays the role of a template. Jacob & Monod, in a definitive review (182) of the problem of the genetic control of "structural" genes, predict the existence and general properties of such an RNA, and have referred to it as "messenger" RNA.

Two lines of evidence have been used in support of the existence of this new type of RNA. The first was based on its rapid rate of synthesis as compared with ribosomal RNA; Volkin & Astrachan (183) found, in phage-infected *E. coli* that the major portion of the incorporated P^{32} was in a non-ribosomal RNA fraction. However, a sufficient amount of information has not been available on the turnover rate, under varied conditions, of ribosomes or sRNA, to enable any definite conclusions to be drawn from such comparisons. Secondly, Volkin and co-workers found that the pattern of P^{32} labeling of the RNA nucleotides was distinctly different in the phage-infected cells (175, 178, 179) as compared with the host cells (184, 185). Moreover, the base composition of the newly formed RNA, determined from the labeling data, reflected the base composition of the DNA of the particular infecting phage, rather than of host DNA, ribosomal RNA, or sRNA. These results provide evidence that the RNA is of a new type (175) rather than being related to sRNA or ribosomal RNA.

The base-ratio data are in accord with, but by no means offer proof for, the hypothesis advanced earlier that the RNA acts as an information carrier from DNA and possibly plays the role of a template in protein synthesis. A cogent argument (186, 187) against the hypothesis that ribosomal RNA plays the role of such a template is that neither the large variation in the molecular weights of proteins nor in the nucleotide composition of the DNA of different microorganisms (188, 189, 190, 191) is reflected in the remarkable homogeneity in size (192, 193, 194) and base composition (188, 191) of the ribosomal RNA. In the more recent work now to be discussed, the new RNA will be termed mRNA rather than "messenger" (182) [or "informational" (195)] RNA, with the hope, however, that future evidence with respect to its function will justify the use of this appellation as well as of the abbreviated form.

Nomura *et al.* (196), by means of sucrose density-gradient centrifugation and starch-gel electrophoresis, obtained strong evidence that the RNA which becomes rapidly labeled with P^{32} after T2 phage infection of *E. coli* is distinct from previously known RNA components of the cell. After centrifugation of the untreated disrupted cell suspension, the bulk of the P^{32} -RNA was found in the ribosomal fraction, although some appeared in other fractions. In contrast, Volkin & Astrachan (183) found most of the P^{32} -RNA in the soluble fraction, probably because of their use of a lower Mg^{++} concentration; electrophoresis experiments (196) indicated that at a lower Mg^{++} concentration most of the P^{32} -RNA is released from the ribosomes. Both the P^{32} -RNA in the ribosomal and in the soluble fraction were distinguished from the

normal components by their base ratios, which were those of T-2 DNA and agreed with those reported by Astrachan & Volkin (184).

The unique nature of the P^{32} -RNA became clearly evident when RNA was prepared from ribosomes by phenol treatment. On electrophoresis, a P^{32} -RNA peak, undetectable spectrophotometrically, was found well ahead of the major portion of the RNA, and only in infected cells. Sedimentation analysis of the ribosomal RNA gave a major P^{32} peak of 8S and two normal unlabeled RNA peaks of 18S and 25S. A P^{32} peak corresponding to the 8S peak was also found in uninfected cells; in contrast, however, the ribosomal RNA peaks were heavily labeled with isotope. The results suggest that mRNA synthesis occurs in both normal and infected cells whereas, in the latter, ribosomal RNA synthesis does not proceed. What is not clear is why, in normal cells, the 8S peak was detectable only by sedimentation analysis, whereas, in infected cells, the peak was detectable by electrophoresis as well.

The soluble P^{32} -RNA (i.e., before phenol treatment) found in infected cells possessed an S value corresponding to that of sRNA (about 4S) but its base ratio was the same as that of the 8S material found in the ribosomes; this suggests the possibility that it may be a precursor or a degradation product of the 8S component. In contrast, the base composition of sRNA, determined by chemical analysis, is the same in normal and infected cells.

The detection of mRNA in normal cells.—The results of a study by Spirin *et al.* (191, 197), based on the statistical correlations between the base compositions of the total RNA and DNA of a variety of normal microorganisms, left room for the presence, in the RNA, of a small fraction having the composition of the homologous DNA [see also Woese (198)]. Experimentally, the detection of mRNA is easier in infected than in normal cells, because, in the former, the synthesis of almost all other RNA appears to come to a halt. Nevertheless, by the use of "nearest neighbor" analysis, and by measuring the specific activities of the 5' as well as the 2'+3' nucleotides of total yeast RNA from cells grown in the presence of P^{32} , and by demonstrating a correlation between these two values, Ycas & Vincent (199) could eliminate nonrandomness as the explanation for the differences in specific activity of the four 2'+3' nucleotides and were able to infer that a fraction of high turnover rate with a nucleotide composition close to that of yeast DNA was present in the total RNA. The fact that the base composition was not exactly that of DNA, together with the number of assumptions that were necessary to calculate these values, demonstrates the desirability of separating and isolating this new type of RNA, if indeed only one type is involved. As an example, in view of the relative length of the P^{32} pulses in experiments in which exponentially growing cells were used, the assumption that no other RNA becomes labeled would appear to be unwarranted.

When rapidly growing cells were slowed down by transfer to a less complete medium (200, 201, 202), synthesis of ribosomes ceased. Inasmuch as

protein synthesis continued normally for a time, it was hoped that mRNA synthesis would continue, as well. Using this "step-down" technique, Hayashi & Spiegelman (203) detected mRNA in *E. coli*, in *Pseudomonas aeruginosa* [in agreement with Astrachan & Fisher (204)], and in *Bacillus megaterium*, after a P^{32} pulse experiment. In the case of *Ps. aeruginosa*, however, the agreement with the base composition of homologous DNA is not very close, and the values correspond almost equally well with those of the bulk RNA; in *E. coli*, the base compositions of DNA and total RNA are so close (percentage of guanine plus cytosine in DNA, 52.0; in RNA, 54.3) that the significance of their similarity in the DNA and the newly formed RNA is questionable. The difference between the composition of DNA and total RNA is greater in *B. megaterium*, and the composition of the mRNA is considerably closer to that of the homologous DNA than to the RNA. It is well to note here that caution must be exercised in the use of purine/pyrimidine ratios in such comparisons, particularly where the base compositions do not agree. The characteristic base equivalence of DNA is also true of sRNA, and, in addition, the presence of both complementary strands of an RNA whose base composition bears no relation to that of DNA is always possible.

Density-gradient centrifugation of extracted RNA provided stronger evidence of the existence of mRNA in normal cells (203). In contrast to infected cells, the P^{32} -RNA was spread throughout the density gradient, some corresponding to the 4S (sRNA) and 16S and 23S peaks (ribosomal RNA), particularly in *Pseudomonas*, and some peaks lying in the 6 to 12S region in which the mRNA of infected cells is found. The analysis of the base ratios of the P^{32} -RNA showed that the (6 to 12S) fractions corresponded well to homologous DNA, while the correspondence decreased with increase of S. This could mean that a considerable amount of ribosomal RNA had been synthesized, despite the use of the "step down" technique. If a P^{32} pulse is "chased" with P^{31} -phosphate, all evidence of mRNA disappears (203); the P^{32} peaks are now coincident with the absorbancy peaks of the usual RNA constituents, thus indicating that some ribosome synthesis does occur after "step down"; and the base ratios of the P^{32} -RNA in the ribosomal peaks have become typically ribosomal. The base ratios in the 4S peak lay between those of sRNA and mRNA. This suggested to the authors a slower rate of breakdown of the already fragmented mRNA.

By keeping the RNA precursor pulses very short, Gros *et al.* (205) hoped, by sucrose density gradient studies, to detect mRNA in normal cells without interference from the remainder of the RNA species which presumably are synthesized much more slowly. In either phage-infected or normal cells, at 10^{-4} M Mg^{++} in which 30S and 50S ribosomes predominate, they found that most of the labeled RNA sedimented at 14 to 16S. However, some label was associated with the "stable" 70S ribosomes [Tissieres *et al.* (206)]. This association is a function of the Mg^{++} concentration, as found by Nomura *et al.* (196), and is reversible.

Studies on RNA purified by the Duponol-phenol procedure showed similar patterns for phage-infected and normal cells. Most of the labeled RNA was 8S, however, and there was no synthesis of ribosomal RNA (16S, 23S). No evidence could be found that the change in S value resulted from RNA breakdown during purification. However, it is known that rapid breakdown of mRNA can occur (195, 207).

It is clear that phage infection of *E. coli* evokes a new kind of RNA. Whether this RNA is really a special kind of phage-specific ribosome, or a free substance which merely enters the pre-existing ribosome, is a crucial problem. The apparent presence of this material in normal cells, and the ease with which it is released from 70S particles at low Mg^{++} concentrations, favor the latter alternative. Additional force is given this view by the results of Brenner *et al.* (187). If bacteria are grown in a C^{13} - N^{15} medium so that all cellular constituents are heavy (i.e., dense), and if all cellular reactions, subsequent to phage infection and P^{32} pulse, are then permitted to take place in a C^{13} - N^{14} medium, so that all new components are light (208), then addition of carrier cells and examination of the pre-infective and post-infective ribosomes isolated by cesium chloride density-gradient centrifugation will show with which particles the newly synthesized P^{32} -RNA is associated. The results showed clearly that the P^{32} -RNA was associated with the heavy (pre-infective) ribosomes and not with the light (new) ribosomes. Moreover, the light ribosomes derived from the carrier cells contained no P^{32} , showing that there was no synthesis of ribosomes after infection. Lowering of the Mg^{++} concentration released the P^{32} -RNA from the 70S particles, in accord with previously discussed observations. In an identical experiment, except that S^{35} -sulfate was used in place of P^{32} -phosphate, evidence was obtained of protein synthesis in pre-infective ribosomes of infected cells, thus making it unnecessary to invoke the direct involvement of DNA (but not excluding it, however) in phage protein synthesis, and therefore leaving room for RNA to act as a carrier of information from phage DNA to the site of protein synthesis.

The DNA-like base composition of mRNA is significant only if it signifies a similar nucleotide sequence. In an experiment based on the recent findings of Marmur, Doty, *et al.* (209, 210), Hall & Spiegelman (211), using cesium chloride density-gradient techniques, succeeded in showing that a DNA-RNA hybrid molecule can be formed from the partly purified P^{32} -mRNA of T_2 phage-infected *E. coli* and H^3 -labeled T_2 -DNA. The formation of the hybrid (composition: 5 DNA:1 RNA) was dependent upon the reacting species being in the single-stranded state and was specific for T_2 -DNA. With normal cells, Spiegelman *et al.* (212) [consult (195)] found that hybridization occurred between 8 to 12S mRNA, from "step down" cultures of *E. coli* or *Ps. aeruginosa*, and exclusively homologous DNA.

The restriction of the formation of DNA-DNA hybrids to DNA strands of similar base composition from closely related species (209, 210) provides

some evidence that at least portions of the two strands of DNA must be complimentary. How much of this reasoning can be extrapolated to DNA-RNA hybrids, or whether the possibility can be ruled out completely that some property of nucleic acids other than complementarity can lead to the formation of such hybrids, is a matter of conjecture at the present time. The finding of a T₂DNA-mRNA hybrid in phage-infected *E. coli* cells [Speigelman *et al.* (213)] cannot be ignored, however, but its interpretation awaits an understanding of the mechanism of DNA-directed RNA synthesis in highly purified enzyme systems [consult (1)]. Attempts have been made to see whether DNA-RNA hybrid molecules appear in the course of the synthesis of RNA in such a system, and some evidence has been obtained that they do not (214).

DNA-RNA hybrids have been found in liver nuclei from embryonic chicks [Venkataraman & Coe (215)], and also in a pea embryo system which is related to chromatin, and which incorporates all four ribonucleotides internally into RNA [Bonner *et al.* (216)]. It was concluded (217) that the DNA and RNA are bound in some fashion other than in a double-stranded helix. It is not known whether the RNA formed in any of these nuclear systems is related to mRNA. However, in a thymus nuclear system which required all four nucleoside triphosphates for RNA synthesis, the base composition of the newly formed RNA reflected the base composition of the RNA of nuclear ribosomes, rather than that of the thymus DNA [Biswas & Abrams (218)]. A subcellular system prepared from a *Pseudomonas*, however, incorporates P³² into RNA, and here the newly formed RNA begins to resemble ribosomal RNA only after extended time intervals [Mizuno *et al.* (219)].

With respect to the DNA-directed synthesis of RNA, Cohen & Nisman (260) have discovered a structure in *E. coli* which incorporates amino acid into protein, synthesizes β -galactosidase, and contains DNA, RNA, and protein. On treatment with DNase, it was destroyed and liberated ribosomes.

It must be emphasized in concluding this section that experimental verification of the role of mRNA as a template and of the precise role of the ribosomes is lacking. While some recent evidence is at hand [Volkin (220)] from experiments with double mutants of *E. coli* (adenineless, arginineless) that the synthesis of mRNA during preincubation with adenine is required for the later synthesis of phage, the precise role of mRNA in this process is unknown. Moreover, the longevity of enucleated *Acetabularia* (168, 169, 170) indicates that if mRNA is indeed present, it does not break down rapidly; and results with *Euglena* clearly indicate that total RNA [Pogo *et al.* (237)] and ribosomal RNA [Brawerman (238)] of different base composition are synthesized concomitantly with chloroplast formation, thus suggesting some type of specificity associated with these particles. A further need for caution is demonstrated by the results of McCarthy & Aronson (239) who have

studied the kinetics of, and the intermediates in, ribosome synthesis. The relation between mRNA and the ribosome precursors (particularly the 4 to 8S material) found by these authors is in need of clarification.

Influence of synthetic polynucleotides on amino acid incorporation.—By use of a 30,000 g supernatant fluid, or washed ribosomes and a 105,000 g supernatant fluid, obtained from *E. coli*, Matthaei & Nirenberg (221) have found that the amino acid-incorporating system is energy dependent, is inhibited by DNase, RNase, puromycin, and chloramphenicol, and is stimulated by an amino acid mixture. In addition (222): (a) at saturating concentrations of sRNA, the addition of phenol-purified homologous ribosomal RNA (comprised of 23S, 16S, and 4S components) or heterologous RNA (yeast, tobacco mosaic virus) exerted a marked stimulatory effect which was energy-dependent, was stimulated by an amino acid mixture, was sensitive to puromycin, chloramphenicol, and RNase (but insensitive to DNase), and required both ribosomes and 105,000 g supernatant fluid; (b) the stimulation could not be observed unless the ribosomes were washed; and (c) RNA was active, but not sRNA.

These observations are in accord with the previously discussed view of the roles of mRNA and the ribosomes in protein synthesis. The inhibition of amino acid incorporation by DNase in the unwashed ribosomal system is in accord with this view and can be attributed tentatively to its inhibition of mRNA synthesis, which accounts for the failure of this nuclease to inhibit the stimulation of incorporation by added ribosomal RNA. Thus, if the function of the ribosome is merely to act as a receptor for "template" RNA, will the Matthaei-Nirenberg system respond to the addition of a relatively simple synthetic polynucleotide? Nirenberg & Matthaei (222) then made the brilliant observation that, when poly U was added to the washed ribosomal system, the incorporation of L-phenylalanine was stimulated 1000-fold. Of 18 C^{14} -amino acids tried, only phenylalanine incorporation was stimulated, and likewise, the incorporation was specific for poly U; no effect was observed with poly A, poly C, poly I, or poly A-U (2:1). Phenylalanyl-sRNA appears to be an intermediate in this process (223), and the other properties of the system are identical to those given above for the washed ribosomal system, with the exception that an amino acid mixture is without effect, in agreement with the observation that poly U stimulates the incorporation of L-phenylalanine alone. Solubility and kinetic studies suggest that the product is polyphenylalanine, but further evidence is necessary to substantiate this point.

Attempts to decipher the RNA-protein code.—Should poly U be acting as a template in the synthesis of polyphenylalanine, this would mean that a sequence (of unknown length) of uridylic acid residues is the code word for phenylalanine. Making this assumption, the laboratories of Ochoa (224 to 227) and of Nirenberg (228) have independently been engaged in attempts at deciphering the code by measuring the stimulation, by synthetic polynucleotide homo- and copolymers, of amino acid incorporation into the TCA-

TABLE I

COMPARISON OF EXPERIMENTALLY DETERMINED NUCLEOTIDE CODE WORDS FOR AMINO ACIDS WITH AMINO ACID REPLACEMENTS OBSERVED IN TMV MUTANTS

Amino Acid Replacement in TMV Mutant	Assigned Code Words	
	Nirenberg <i>et al.</i> **	Ochoa <i>et al.</i>
Asp*→Ser (233, 235)		UAG, UAA(UAC)→UUC‡
Asp*→Ala (234, 235)	UCG	UAC→UGC
Asp*→Gly (235)		UAG→UGG
Asp →Lys (234)		UAG→UAA‡
Arg →Gly (234)	UCG	UCG→UCG‡
Glu†→Gly (234, 235)	UCG→U ₂ G _y	UAG→UGG
Glu →Val (235)	UCG→UUG	UAG→UUG‡
Ileu →Val (235)	U ₂ A _y →UUG	UUA→UUG
Leu →Phe (235)		UUC→UUU
Pro →Leu (234, 235)		UCC→UUC
Pro →Ser (235)		UCC→UUC
Ser →Leu (235)	UCG→UUG, UUC→UUC‡	UUC→UUC‡
Ser →Phe (234, 235)	UCG→UUU‡, UUC→UUU	UUC→UUU
Thr →Ser (233, 234)		UCC→UUC
Thr →Met (235)	U ₂ G _y	UAC(UCC)→UAG‡
Thr →Ileu (235)		UAC→UAU
Tyr →Phe (234)	UUA	UUA→UUU‡
CySH	U ₂ G _y	UUG
Try	U ₂ G _y	UGG

* Aspartic acid or asparagine

** The assigned letters have been adjusted to conform with a triplet code.

† Glutamic acid or glutamine. Code word for glutamine not available experimentally.

‡ Disagreement with amino acid replacement.

insoluble material of well-washed ribosomal systems from *E. coli*. The nucleotide composition of the code words (but not the sequence, which is still unknown) is given in Table I.

The criteria used in deciding which code letters (but not their sequence) were to be assigned to an amino acid have been extracted from the various logical manipulations carried out (224 to 227) and are summarized as follows: (a) The nucleotide sequences of the copolymers was considered to be random; (b) A triplet (i.e., three-nucleotide) code for all amino acids was assumed; (c) Polymers which showed essentially no ability to stimulate amino acid incorporation were eliminated from consideration; (d) Where a single homopolymer stimulated one amino acid only, the amino acid was assigned the appropriate triplet letters, e.g.: 3U for phenylalanine. Note that the incorpo-

ration of phenylalanine would also be expected to be stimulated by copolymers if the frequency of occurrence of 3U triplets was sufficiently high; (e) Where an amino acid was clearly stimulated by a single two-nucleotide copolymer, and by no homopolymer, e.g., serine by poly U-C, the decision as to whether the letters are 2U-C or U-2C was made in the following way: the ratio of the stimulation of the incorporation of phenylalanine to that of serine (4.4:1) was compared with the calculated ratio in the polymer of the amount of the phenylalanine triplet to the amount of each of the other possible triplets (3U:2U-C is 5:1; 3U:U-2C is 25:1). That triplet was selected whose ratio to 3U in the polymer corresponded to the ratio of the incorporation of the amino acid in question to that of phenylalanine. Thus, the correspondence of 5:1 and 4.4:1 would lead to the assignment of the code letters 2U-C to serine; (f) If two or more two-nucleotide copolymers, but no homopolymer, stimulated incorporation of an amino acid, maximal stimulation was tentatively taken as the significant result; (g) Three-nucleotide copolymers were treated in a fashion entirely analogous to two-nucleotide copolymers.

These criteria and the results of the experiments deserve some comment. If the assumption that the code is a triplet one is not correct, the quantitative composition of the code words would change, but the kinds of letters should remain the same. Crick *et al.* (229) have reported results based on genetic experiments on T₄ phage which, together with the results on amino acid replacements in TMV mutants (233, 234, 235), suggest a nonoverlapping triplet code of the comma-free type; the code is "read" by starting at a fixed point, say the free 3' end of the polynucleotide, and working along the sequence of bases, three at a time. An operation of this kind is in accord with the findings of Bishop *et al.* (230) and of Dintzis (231) on the sequential synthesis of hemoglobin (starting at the N-terminal end), although other mechanisms of assembly are not excluded.

With respect to item (f) above, such findings can be accounted for theoretically if more than one code word can specify an amino acid (degenerate code). In the decision of the proportions in which the code letters occur in a triplet [item (d) above], difficulties arose in certain cases where no ratios showed reasonable correspondence. The authors (226) attribute this to a slight nonrandomness in the base sequence of the polynucleotide which would make the calculated frequency distribution of the various triplets incorrect, but this nonrandomness has yet to be demonstrated experimentally. Moreover, to resort to such an argument of necessity raises the question of whether ratios which do agree well, do so fortuitously.

It may be asked whether the code letter assignments would remain constant if the experimental conditions (e.g., concentration of polynucleotide, or composition of amino acid mixture) were changed. The answer to this question will have to be known before the validity of the present assignments can be fully assessed.

It is remarkable that the code words for all the amino acids tested thus far contain uridylic acid. Inasmuch as no polymers in which uridylic acid is absent have been tested [because of the hazard that short nonphenylalanine polymers might be soluble and therefore undetected by the methods used (223)], the question may be raised as to whether this ubiquity of uridylic acid is fortuitous, or whether it results from extensive degeneracy of the code, or from the operation of some special mechanism. One also wonders whether inconsistencies may be encountered should a four-nucleotide copolymer be tested.

Other considerations bearing on the validity of the assigned code words are of interest. Making use of the assumption, again, that the code is of the triplet type, a consequence of the conclusion that 3A is not a code word is that 3A triplets should not occur in the RNA template. A sequence of 5A units would be sufficient to indicate such a triplet; any less could be construed to mean that the sequence of A units belongs to two vicinal triplets, with no 3A sequence acting as a code word. Direct evidence for or against the existence of such a sequence in any RNA is lacking. However, if "template" RNA and mRNA are assumed to be the same substance, then the presence of a 5A sequence in mRNA should be indicated by a 5T sequence in DNA. Such a sequence has, in fact, been demonstrated to occur [Spencer & Chargaff (232)], and constitutes an argument, if not evidence, against the validity of the code assignments, at least with respect to poly A. The argument is weakened, however, by the following consideration. Both strands of DNA are active in the synthesis of two strands of RNA complementary to one another, as shown by the similar base composition of the two. The RNA strand which is complementary to the DNA strand containing the 5T sequence would have a 5A sequence. However, if it is assumed that the complementary strands of RNA could not both be templates (in view of the one gene, one enzyme theory, as well as the sheer difficulty in the construction of a suitable code), it is possible that the 5A strand is the nonsense strand and, therefore, the detection of its presence is not an argument against the assignment of no function to the 3A triplet.

In seeking independent support for these assigned code letters, it is instructive to compare them with observed amino acid replacements in TMV mutants produced by nitrous acid [Tsugita & Fraenkel-Conrat (233); Tsugita (234); von Wittmann (235)]. In considering these exchanges, it is helpful to recall that deamination of the bases of TMV-RNA with nitrous acid, and subsequent infection of the plant, leads to the following base transformations (236) in newly replicated virus: cytosine to uracil, adenine (via hypoxanthine) to guanine, and guanine (via xanthine) back to guanine; uracil is not attacked by nitrous acid. The unproven assumption must be made, however, that no other transformations result from the nitrous acid treatment. An inspection of Table I shows that the agreement is only fair. It is evident that further work is necessary to evaluate the validity of the detailed assign-

ment of each individual word of the code, as well as of the assumption that it is the same throughout nature for every amino acid. However, the extent of agreement and the internal consistency are sufficiently good to leave little cause for doubt that the general approach is a valid one.

OTHER SYSTEMS

A number of systems exist which appear to incorporate amino acids into protein by a pathway which differs, at least in part, from that discussed previously in this review. Although space limitations prevent extensive discussion of any of these systems [see (7)], the recent unusual turn of events taken by the experiments on mitochondria merits a short account of this problem.

Since the discovery [McLean *et al.* (240)] that liver and muscle mitochondria are active in protein synthesis, the mitochondria of a variety of other tissues and organisms [for citations, see (241, 242, 243)] have been implicated in this role. The incorporation process is energy-dependent and does not appear to require complex factors (such as RNA or protein) from the soluble fraction of the cell [Roodyn *et al.* (243); Truman & Korner (244); Skinner *et al.* (245)]. RNase does not inhibit the amino acid incorporation (240), despite its promotion of the degradation of a minor part of the mitochondrial RNA (243). However, a nonsedimentable system derived from heart mitochondria [Kalf & Simpson (250)] is sensitive to RNase. The incorporating activity, when related to the RNA content, is of the same order as that of liver ribosomes (244). The addition of an amino acid mixture stimulates PP^{32} -ATP exchange [Reis *et al.* (246)] and, upon individual analysis, at least two activating enzymes were found to be present [Craddock & Simpson (241)].

A great deal of evidence has accumulated to indicate that the observed amino acid incorporation into protein is an inherent property of the mitochondria (240, 243, 244, 246) and that it represents true incorporation into peptide bonds [(240, 243) and see (249)]. However, the evidence obtained for amino acid incorporation into a specific protein, cytochrome-*c* [Bates & Simpson (248)], cannot be substantiated [Simpson, Skinner & Lucas⁵ (242)] and should be completely discounted.

The failure to find amino acid incorporation into mitochondrial cytochrome-*c* has been confirmed independently [Roodyn *et al.* (249)] and, in addition, no incorporation could be found into another mitochondrial enzyme, malic dehydrogenase (249). In the light of these findings, the question arises as to what types of proteins are being made in mitochondria. Roodyn *et al.* (243, 249) have found that the bulk of the labeled amino acid appeared in a detergent-insoluble fraction rich in phospholipid, RNA, and succinoxidase activity, and, upon further fractionation, it was found that the major

⁵ With the aid, through extensive correspondence, of Dr. V. M. Craddock to whom we are indebted.

part of the incorporation was into a relatively insoluble lipoprotein. The mitochondria, being essentially a membranous structure, may be yet another example of lipoprotein and membrane systems which are capable of incorporating amino acids into protein. Such cell-free systems have been obtained from hen oviduct [Hendler (251, 252) and Hendler & Love (253)], from *E. coli* [Nisman (254); Spiegelman (255)], from a *Pseudomonas* [Mitsui & Yoshida (258)], from *B. megaterium* [Godson & Hunter (256, 257)], and from other organisms (consult citations in above publications). Mitsui, in a preliminary communication (259), has reported that such a bacterial membrane system can effect the incorporation of amino acids into cytochrome-*c*. Ribosomes have been found in close association with the membrane fraction in certain cases. They could be liberated by treatment with deoxycholate or by sonic vibration, and in the investigations of Nomura *et al.* (196), they were found to have sedimentation coefficients of 30S and 50S. However, there appears to be insufficient evidence from the results of Rendi (261) to substantiate his claim of the presence of ribosomes in mitochondria.

The results of Fletcher & Sanadi (262) on intact rats would seem to indicate that all major components of the mitochondria are synthesized at the same rate. This could be interpreted as meaning that these particles are produced by some other system and once formed, the components no longer turn over. This interpretation is inconsistent with the observed amino acid incorporation into mitochondrial protein *in vitro*, unless this incorporation represents a specialized process possibly characteristic of membranes.

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LIPID METABOLISM^{1,2}

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The recent expansion and diversification of research in the field of lipid chemistry and metabolism have made it mandatory to limit the reviewer's survey to selected topics. This review presents in detail various known facets of fatty acid synthesis and summarizes the annual accomplishments in other selected areas. Although accomplishments in fatty acid synthesis have been reviewed annually, no unified and complete assessment of the progress and problems in fatty acid biosynthesis has been presented previously. During the past few years this area of research has been particularly active and warrants consideration. Therefore, the presentation of a more detailed account of the current status of the synthesis of fatty acids seems particularly timely and useful.

Several excellent review articles have appeared recently covering aspects of lipid metabolism not included in this review. The reader's attention is directed to the following review articles: *Pancreatic Lipase*, by Desnuelle (46); *The Metabolism of 2-Carbon Compounds by Microorganisms*, by Kornberg & Elsdon (101); *The Metabolism of Adipose Tissue in vitro*, by Vaughan (197); and *Biosynthesis of Cholesterol*, by Popják & Cornforth (142).

TRIGLYCERIDES

Fatty acid content.—With the advent of new methodology and techniques for the separation and purification of the various lipids, exact analytical information has been obtained on the content and distribution of various lipid components in cellular subfractions. Getz & Bartley (59) studied the distribution of fats and their constituent fatty acids in subcellular organelles of rat liver. The total lipids from nuclei, mitochondria, fluffy layer, microsomes, and supernatant fluid were found to comprise 15.8, 23.4, 24.3, 30.9, and 11.2 percent of the dry weight of the respective tissue fractions. The whole-liver pulp of the same animals contained 23 per cent lipid. Getz *et al.* (60) fractionated the rat liver lipids into cholesteryl esters, triglycerides, serine phosphatides, ethanolamine phosphatides, inositol phosphatides, lecithin, and sphingomyelin. They found that over 50 per cent of the lipid

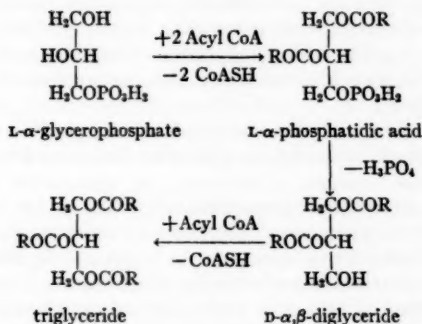
¹ The survey of the literature pertaining to this review covers the period from November 1960 to October 1961.

² The following abbreviations will be used in this paper: CoA (coenzyme A); CoASH (reduced coenzyme A); Pi (inorganic phosphate); NAD (nicotinamide adenine dinucleotide); NADH₂ (nicotinamide adenine dinucleotide, reduced form); NADP (nicotinamide-adenine dinucleotide phosphate); NADPH₂ (nicotinamide adenine dinucleotide phosphate, reduced form).

phosphorus was contained in the phosphatidyl choline fraction and most of the neutral lipid was in the form of triglycerides. The fatty acid content of each of the lipid fractions was assayed by gas-liquid chromatography. The neutral lipid fraction had a high content of palmitate and unsaturated C_{14} fatty acids, whereas phospholipids had high stearate and C_{20} - C_{22} polyunsaturated fatty acids. MacFarlane *et al.* (121) obtained an essentially similar distribution of fatty acids in phospholipids and neutral lipids extracted from mitochondria and microsomes of rat liver cells. The C_{20} and C_{22} unsaturated fatty acids made up 25 and 40 per cent of those present in the phosphatidyl choline and phosphatidyl ethanolamine, respectively. In this regard, various reports described the fatty acid composition of human fatty liver (186), human depot fat (95), and the aortal fat of subjects with atherosclerosis (183). Human depot fat was found to contain 42 to 51 per cent oleic, 21 to 30 per cent palmitic, 5 to 8.5 per cent palmitoleic and stearic, 5 to 8 per cent linoleic, and less than 3 per cent fatty acids with more than two double bonds; a significant amount of odd numbered and branched-chain fatty acids was also reported (95).

The partition of saturated and unsaturated fatty acids within the triglyceride molecule was examined by Savary & Desnuelle (154) and by Mattson & Volpenhein (129) in glycerides of vegetable oils and by Savary *et al.* (153) in glycerides of lymphatic chylomicra of rat. The structure of the triglyceride was determined by hydrolysis with pancreatic lipase, known to hydrolyze triglycerides to free fatty acids and 2-monoglycerides; fatty acid composition was determined by gas-lipid chromatography. It was found by both groups (123, 129, 154) that short-chain fatty acids as well as palmitic and stearic acids were preferentially esterified at the 1 and 3 positions. On the other hand, the 2 position of the triglyceride molecule contained a high proportion of the unsaturated fatty acids (oleic, linoleic, and linolenic acids). The relationship between the composition of ingested triglycerides and those present in chylomicra was studied with the aid of pancreatic lipase (153). When rats were fed triglyceride containing characterized fatty acids (saturated or unsaturated) at the 2 position, large proportions of these acids were located at the same position in the lymph triglycerides. On the other hand, when rats were fed free fatty acids obtained by saponification of the same triglycerides, the structure of the lymph glycerides was quite different. These observations indicated that intraluminal lipolysis of glycerides was not complete and the intestinal mucosa absorbed both free acids and partial glycerides.

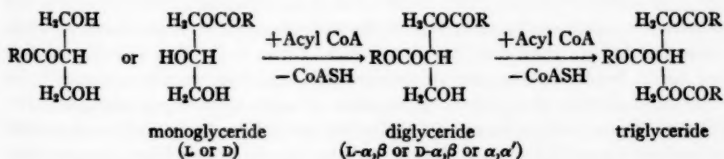
Biosynthesis of triglycerides.—Shapiro *et al.* (158) studied the incorporation of C^{14} -labeled fatty acids into the triglycerides of adipose tissue and found the isolated triglycerides to be labeled in all three positions. Steinberg *et al.* (175) independently studied the synthesis of triglycerides in homogenates of adipose tissue. They found an absolute requirement for ATP and CoA for triglyceride synthesis. α -Glycerophosphate stimulated the incorporation of labeled fatty acids into the glycerides. The findings of both groups

SCHEME I. Biosynthesis of Triglyceride from L- α -Glycerophosphate.

are in accord with the pathways proposed by other workers for triglyceride synthesis in the liver (166, 172, 192, 213) as shown in Scheme I.

Adipose-tissue homogenates (175) catalyzed the incorporation of C^{14} -labeled α -glycerophosphate, while, after 30 min of incubation, the neutral lipid fraction contained about 70 per cent of the total lipid- C^{14} .

Clark & Hübscher (35, 36, 37, 84) presented evidence to show that mitochondrial preparations isolated from both rat and rabbit intestinal mucosa were able to esterify monoglycerides with C^{14} -labeled fatty acids yielding di- and triglycerides. Adenosine triphosphate and monoolein were absolutely required for the synthesis, while CoA was partially required. The ATP requirement for synthesis was taken as indicating the exclusion of lipase-catalyzed esterifications. Using purified particulate preparations, Clark & Hübscher (36, 84) were unable to replace monoolein by α -glycerophosphate or glycerol but other monoglycerides (α -monopalmitin and monolaurin) and diglycerides (α,β -dipalmitin and α,α' -distearin) were active. Glycerides containing saturated fatty acids were maximally active when the fatty acid chain length was between C_{10} and C_{12} . The relative activities of the substrates may be a reflection of the ability of these glycerides to form stable and highly dispersed emulsions rather than true enzymatic specificity. Both the α,α - and α,β -diglycerides were esterified by these mucosal preparations, suggesting the nonspecific nature of the enzyme(s) for either the α - or the β -



SCHEME II. The Biosynthesis of Triglycerides from Monoglyceride.

hydroxyl group. This did not exclude the possible presence in the mitochondria of two distinct enzyme systems, each of which is responsible for either the α - or β -position on the glyceride. These observations led Clark & Hübscher (35) to propose a new scheme for the synthesis of triglycerides in mucosa (Scheme II).

The enzyme catalyzing the acylation of monoglycerides by acyl CoA was called monoglyceride transacylase (84). The new system has been found in the mitochondrial fractions of rat liver, rat and rabbit small intestinal mucosa, and in supernatant preparations from pig kidney, rabbit pancreas, and rat brain (85). The general distribution in various tissues signified an important metabolic role for this system in triglyceride synthesis. Location of the monoglyceride transacylase in the intestinal mucosa points to its possible importance in the resynthesis of triglycerides during intestinal absorption. The monoglycerides as well as the free fatty acids derived from lipase action on triglycerides can be used as substrates for the intestinal mitochondria yielding the triglycerides found in the chylomicra. The fact that β -monoolein obtained by hydrolysis of triolein with pancreatic lipase was three to five times more active than α -monoolein as an acceptor substrate (85) may be considered as an added indication for the role of this system in intestinal absorption. One of the main reasons for triglyceride hydrolysis in the gut and subsequent resynthesis by the mucosa appears to be that of facilitating absorption of these complex molecules by breaking them down to smaller, more dispersible molecules. The price for this efficiency in absorption is the expenditure of ATP for mucosal resynthesis of triglycerides. Scheme II is more economical, as far as the expenditure of energy is concerned, than is its counterpart, Scheme I. Synthesis via pathway I consumes two ATP molecules (needed for the activation of the two fatty acids) while synthesis via pathway II requires four ATP molecules (three molecules for the activation of fatty acids and the fourth ATP for the phosphorylation of glycerol).

PHOSPHOLIPIDS

Cellular distribution.—The distribution of the various phospholipids in subcellular particles of rat liver and brain was reported by several workers (13, 40, 41, 67, 121). Collins & Shotlander (41) examined the phospholipid distribution in rat liver mitochondria and microsomes by the countercurrent distribution of dinitrophenylated and methylated derivatives. These results were essentially similar to the findings of MacFarlane *et al.* (121), as well as those of Marinetti *et al.* (124), in that the phospholipids of mitochondria and microsomes were similar except that the mitochondria contained significantly more cardiolipin than did the microsomes. Biran & Bartley (13) found that rat brain homogenates and mitochondrial and microsomal phospholipids contained similar proportions of lecithin, phosphatidylserine, phosphatidylethanolamine, and corresponding plasmalogens. The fatty acid composition of each of these lipid classes showed only minor variations from one centrifugal fraction to another. In comparison with lipids from other tissues (rat

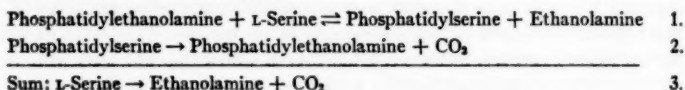
liver, ox heart mitochondria) (41, 49, 121, 124) brain phospholipids are remarkable for their high content of saturated fatty acids (40 to 60 per cent) and low proportion of polyenoic fatty acids (10 to 25 per cent). Rat brain mitochondrial phospholipid was found by Biran & Bartley (13) to contain 32 per cent lecithin, 50 per cent phosphatidylserine and phosphatidylethanolamine, 13 per cent phosphoinositide, 2 per cent polyglycerophosphatide, and 17 per cent plasmalogens (serine and ethanolamine). No choline plasmalogens were found.

Phosphatidic acid.—Available evidence derived from various sources pointed to an increasingly important role for phosphatidic acid in the metabolism of the phospholipids and possibly in active transport. Kornberg & Pricer (100) were the first to describe the biosynthesis of phosphatidic acid from L- α -glycerophosphate and long-chain fatty acyl CoA's in the presence of a particulate preparation from rat liver. Kennedy and co-workers (92, 141) presented evidence to show that phosphatidic acid is a precursor of phospholipids and triglycerides. Hokin & Hokin (78) postulated a role for phosphatidic acids in active transport and suggested an alternative pathway for synthesis in brain tissue involving the phosphorylation of D- α , β -diglyceride by ATP (77). Despite the aforementioned evidence, earlier workers (44, 125) were unable to detect phosphatidic acid in rat heart or liver or in guinea pig brain. More recently, however, Hübscher & Clark (83, 86) were able for the first time to isolate phosphatidic acid from the liver of ox, pig, and rat. The isolated phosphatidic acid contained an unusually high percentage of unsaturated fatty acids (75 to 89 per cent oleic and linoleic acids); 6 to 7 per cent of the isolated phospholipid seemed to be present as plasmalogen.

The dephosphorylation of phosphatidic acid by a specific phosphatase was shown by Kennedy and his co-workers (92, 141) to be one of the steps in the synthesis of triglycerides and phospholipids. Smith *et al.* (166) were the first to describe particulate preparations from chicken liver which catalyzed the dephosphorylation of phosphatidic acid. These observations were confirmed by Rossiter & Strickland (152) and were extended by Hokin & Hokin (79), who demonstrated phosphatidic acid phosphatase activity in deoxycholate extracts of brain microsomes. Coleman & Hübscher (38) reported the presence of this enzyme in the kidney, intestine, brain, and liver of pig, ox, guinea pig, rat, and rabbit. They also showed that 60 per cent of the total phosphatase activity was located in the microsomal fraction of the cell. After subjecting the microsomes to autolysis for three days, digestion with ribonuclease, and then treatment with butanol, Coleman & Hübscher were able to solubilize the phosphatidic acid phosphatase without loss of activity. The behavior of the enzyme towards various detergents, organic solvents, and lipases suggested to them that it may be a lipoprotein.

Decarboxylation of L-serine to ethanolamine via phospholipids.—In 1955, Kennedy and co-workers (92) established the role of cytidine nucleotides in the *de novo* synthesis of phospholipids catalyzed by rat liver mitochondrial preparations. Mg^{++} markedly stimulated the synthesis of phosphatidyl-

choline, phosphatidylethanolamine, and phosphatidylserine, whereas Ca^{++} was inhibitory. Recently, Dils & Hübscher (47) described the incorporation of labeled choline into the phospholipids of rat liver microsomes in presence of Ca^{++} and glutathione. The addition of ATP, CoA, and cytidine monophosphate did not effect this incorporation. They attributed these results to an exchange with the choline moiety of the microsomal phospholipids rather than *de novo* synthesis. Similar results were reported by Borkenhagen *et al.* (19) for the Ca^{++} -activated incorporation of labeled L-serine into phosphatidylserine, and by Borkenhagen *et al.* (19) and Artom (6) on the incorporation of labeled ethanolamine into phosphatidylethanolamine. Ethanolamine and L-serine were demonstrated to compete with each other for the same enzyme site, whereas D-serine and choline did not displace ethanolamine or L-serine from the enzyme nor did they incorporate into the phospholipids under these conditions (19). The same authors found that, when L-serine-3- C^{14} was added to the reaction mixture, the principal of C^{14} labeling was recovered in phosphatidylethanolamine and in phosphatidylserine. This observation led to the discovery of the following decarboxylation sequence of serine to ethanolamine:

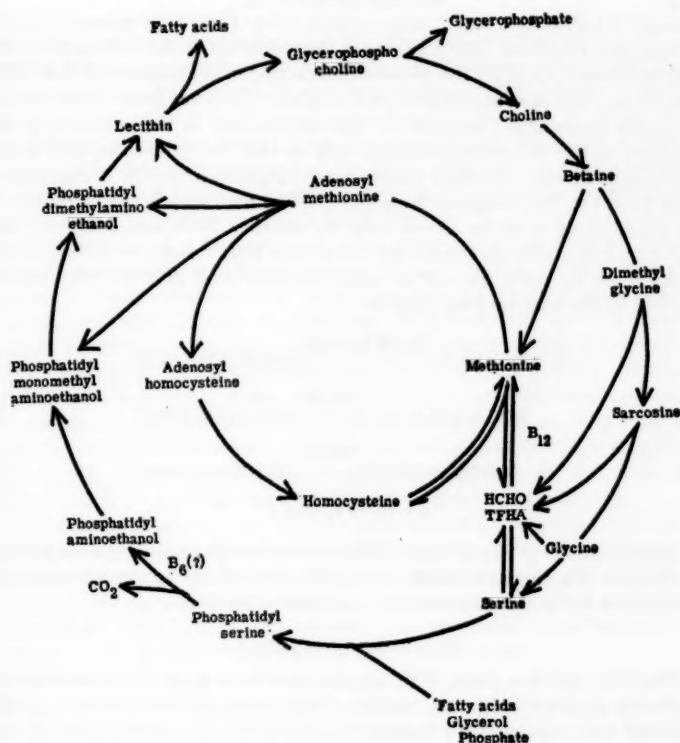


The decarboxylating enzyme was present in mitochondria and was strongly activated by certain organic solvents such as toluene. Neither free serine nor phosphoserine was found to be an intermediate in decarboxylation of phosphatidylserine. Thus, the conversion of serine to ethanolamine is not accomplished by direct decarboxylation, as was expected (26, 139, 214), but via the intermediate formation of phospholipids (phosphatidylserine and phosphatidylethanolamine).

Conversion of ethanolamine to choline.—Recent evidence (26, 65, 214) on the conversion of ethanolamine to choline strongly indicated the involvement of the corresponding phosphatides as substrates. Thus, a phosphatidylethanolamine formed by the decarboxylation of phosphatidyl serine is methylated stepwise by S-adenosylmethionine to form phosphatidylcholine. The reaction is catalyzed by rat liver microsomes and it involves the direct transfer of the methyl group from S-adenosylmethionine to phosphatidylethanolamine to form phosphatidylmonomethylaminoethanol, which then accepts another methyl group to form phosphatidyl dimethylaminoethanol. The latter compound is further methylated by S-adenosylmethionine to form lecithin. Choline can then be produced by the hydrolysis of lecithin.

No additional cofactors are required for this reaction, which appears to take place by the direct transfer of methyl group from the donor to the phosphatidylethanolamine acceptor.

The following metabolic chart (Scheme III) has been advanced by Bremer *et al.* (26) to illustrate the relationship between phosphatide and



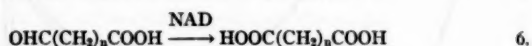
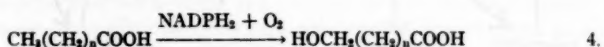
SCHEME III. The Relationship between Phosphatides and Methyl Group Metabolism.

methyl group metabolism. Most of the reactions shown in the diagram are well known and all of them have been demonstrated with *in vitro* systems (29), although the exact mechanism in some cases is not well understood.

FATTY ACID OXIDATION

ω -Oxidation of fatty acids.—The formation of dicarboxylic acid in animals after feeding nonanoic, decanoic, and undecanoic acids was first observed by Verkade & Van Der Lee in 1934 (198). This type of oxidation was called ω -oxidation. Interest in this type of oxidation is increasing, and several recent studies with isolated enzyme systems have been reported. Robbins & Reese (151) reported the oxidation of monocarboxylic acids, having eight to twelve carbon atoms, to their corresponding dicarboxylic acids in the pres-

ence of NADP, NAD, Mg^{++} , and a combination of hog liver microsomes and supernatant fractions. The first step is the oxidation of decanoic acid to 10-hydroxydecanoic acid by the microsomal fraction in the presence of $NADPH_2$ and oxygen. The enzyme responsible for this catalysis has been named omega fatty acid oxygenase (reaction 4). The second step in the sequence is the oxidation of the 10-hydroxydecanoic acid to the 19-oxodecanoic acid by an NAD-requiring enzyme which was isolated and purified by Mitz & Heinrichson (135) from hog liver extracts (reaction 5). The final step is the oxidation of the oxodecanoic acid to sebacic acid by another NAD-requiring enzyme. The dehydrogenases are specific for the omega hydroxy or oxo acids of chain length C_9 to C_{11} only. Shorter or longer member acids of this series are not oxidized by the enzyme preparations.



n is either 7, 8, or 9.

Wakabayashi & Shimazono (200) reported similar requirements for the ω -oxidation of sorbic acid amide to muconic acid amide by a combination of microsomes and supernatant fractions of guinea pig livers.

FATTY ACID SYNTHESIS

Until the past few years, little progress had been made in elucidating the enzymatic steps by which fatty acids are built up from acetate in living cells. Although experiments with isotopic tracers in intact animals greatly enlarged the store of information concerning many aspects of fatty acid metabolism, they provided little information about the intermediate stages in such metabolism. A wealth of literature indicates the fact that fatty acids are formed by multiple "head-to-tail" condensation of acetate and that, during oxidation, fatty acids are quantitatively converted to acetate. Nevertheless, the active form of the fatty acids and the nature of the intermediates remained a mystery for over fifty years. The discovery of the acetylation coenzyme (coenzyme A) by Lipmann (114) and Nachmansohn & Berman (137) and the identification of the "active C_2 unit" as the acetyl thioester of coenzyme A (acetyl CoA) (119) were prerequisites to the isolation and characterization of the various intermediates involved in fatty acid oxidation (68, 118). These intermediates are linked to coenzyme A and, therefore, are present in amounts at most equivalent to the pool of catalytic CoA. Thus, the solubilization and isolation of the various enzymes involved were mandatory to the understanding of the chemical nature of these intermediates. The sequence of reactions involved in the β -oxidation of fatty

acids was essentially identical to Knoop's (99) original formulation, with the exception that acyl CoA derivatives were involved instead of the free acids as originally postulated. Each of the enzymes involved in this reaction sequence has been prepared in a highly purified state and each step was shown to be reversible. These observations led to the postulate that fatty acid synthesis is the exact reversal of β -oxidation (115). This concept was generally accepted by the biochemical community, despite the lack of direct evidence and the earlier observations of Gurin and his colleagues (24, 25, 50, 196) on the possible presence of a distinct system for synthesis of fatty acids. Experimental evidence supporting fatty acid synthesis via "a modified scheme for reversal of β -oxidation sequence" came from two independent observations. The first was the discovery by Langdon (110) that crotonyl CoA can be reduced by NADPH₂ in the presence of an enzyme found in mitochondria (157), the second was the demonstration by Wakil *et al.* (203, 208) that mitochondria can catalyze the elongation of fatty acyl CoA derivatives by the successive addition of acetyl CoA in the presence of NADPH₂ and NADH₂. Since this system is located on the mitochondria and is concerned with the elongation of fatty acids, the name "mitochondrial" or "elongation system" for fatty acids has been proposed for this series of reactions.

Another pathway for the *de novo* synthesis of fatty acids from acetyl CoA has recently been described (64, 145, 146, 210) as being independent of the enzymes of the β -oxidation sequence, and not associated with mitochondrial fraction of the cell. Malonyl CoA was demonstrated to be a key intermediate (202, 206) in this system. The term "non-mitochondrial" or "malonyl CoA" pathway was used to define this system.

Mitochondrial or elongation system for fatty acid synthesis.—Recently, Wakil *et al.* (203, 208) reported the elongation of fatty acids in a mitochondrial system by the successive addition of acetyl CoA to intermediate chain length fatty acyl CoA's (C₁₂, C₁₄, C₁₆). Similar results were reported earlier by Stumpf & Barber (182) in mitochondria derived from avocado mesocarp, though the full significance of the system was not appreciated. More recently, Hulsmann (82) reported on a similar system in rat or rabbit sarcosomes. When rat liver, beef liver, or heart mitochondria were incubated anaerobically with ATP, NADH₂, and NADPH₂, 10 to 20 per cent of the acetyl-1-C¹⁴ CoA was incorporated into long-chain fatty acids. C¹⁴-Malonyl CoA did not substitute for acetyl CoA and ATP in this system.

The role of ATP in this system relates to the activation of endogenous fatty acids to the fatty acyl CoA derivatives, since various intermediate chain length fatty acyl CoA's eliminate the ATP requirement. Butyryl CoA and hexanoyl CoA are poor substrates, whereas longer-chain fatty acyl CoA derivatives (C₈CoA to C₁₆CoA) are excellent substrates. Soluble extracts of mitochondrial acetone powder are active on acyl CoA esters between C₈ and C₁₂ chain length. Mitochondria or its subparticles are also active on longer-chain acyl CoA derivatives (C₈CoA to C₂₀CoA).

The main fatty acid synthesized by both soluble extracts and particles

was shown to be the homologue of the fatty acyl CoA containing two more carbon atoms than the original acyl CoA. The carboxyl group of the higher homologue contained most of the radioactivity when acetyl-1- C^{14} CoA was used (203).

The existence of both mitochondrial and malonyl CoA systems explains the puzzling observation by many workers (93, 98, 122, 150, 156, 167, 179, 180, 218) that the mode of incorporation *in vivo* of acetate into stearate appears to be significantly different from its incorporation to palmitate. In stearate, the radioactivity derived from C^{14} -acetate appears to be concentrated in the first two carbons, whereas in palmitate the radioactivity was equally distributed throughout the entire molecule. Zabin (218) found that the carboxyl group of stearic acid synthesized by rat liver slices from acetyl-1- C^{14} contained relatively higher C^{14} than the rest of the carbon atoms of the molecule, whereas the remaining C^{14} in carbon atoms 3 to 18 of stearic acid appeared to be uniformly distributed along the chain. Palmitic acid which was simultaneously isolated from liver slices showed isotope concentration uniformly distributed along the entire carbon chain. These results suggest that the addition of the last two carbon atoms to palmitate (C_{16}) to form stearate (C_{18}) is accomplished by a mechanism different from the multiple condensation of the C_2 -units to form the C_{16} acid and to form C_{18} acid and may account for the variation of the isotope ratio in carbons 1 and 2 of C_{18} acid as compared to the remaining carbon atoms.

The formation of both saturated and unsaturated C_{20} acids by this system was demonstrated with C^{14} acetyl CoA and either stearyl CoA or oleyl CoA as substrates in the presence of $NADH_2$ and $NADPH_2$; the corresponding C_{20} acids were identified by gas-liquid chromatography (204). The C_{20} acids formed by the addition of acetyl CoA to oleyl CoA were shown to retain the double bonds, presumably between the same carbon atoms as in oleic acid, although no such characterization was attempted. The wide substrate specificity of the elongation system may include the elongation of polyunsaturated acids such as linoleic, linolenic, and others. Thus, the *in vivo* experiments of Mead and his co-workers (131, 132, 133, 173, 174) indicating synthesis of arachidonic acid from C^{14} -acetate, linoleic, and linolenic acids may also be attributed to the mitochondrial enzymes. According to Mead, linoleic acid is dehydrogenated to γ -linolenic acid, which then adds acetyl CoA to homolinolenic acid; the latter acid is then subsequently dehydrogenated to arachidonic acid. Direct experimentation is needed in order to verify the catalysis of these reactions by mitochondria, at least as far as the elongation of the unsaturated C_{18} acid is concerned.

The biosynthesis of the C_{24} acids appears to be accomplished by either the elongation of shorter-chain acids (C_{16} , C_{18} , etc.) or by a *de novo* synthesis from acetate. Fulco & Mead (56) reported the incorporation of C^{14} -acetate into the rat brain C_{24} acids, i.e., lignoceric, nervonic, cerebronic, and 2-hydroxynervonic acids. Lignoceric acid had a specific activity 150 times greater than the average specific activity of the body fatty acids. Studies on the dis-

tribution of the radioactivity in the carbon atoms of the C_{24} acids revealed that lignoceric acid was formed by a *de novo* synthesis from acetate, whereas nervonic acid was formed by the elongation of oleic acid. The site of synthesis of these acids is not known as yet, and further information is necessary in order to implicate either the mitochondrial, the malonyl CoA system, or both, in their synthesis.

The exact steps and the enzymes involved in the elongation of fatty acid are not known as yet. From the nature of the requirements of the system, namely an acyl CoA, $NADH_2$, and $NADPH_2$, and the association of these enzymes with mitochondria, a possible involvement of the enzymes of fatty acid oxidation may be suggested. The elongation system may be similar to the reconstituted system of Seubert *et al.* (157), which, in the presence of the purified enzymes of the β -oxidation cycle (thiolase, β -hydroxyacyl dehydrogenase, and enoyl hydratase) plus $NADPH_2$ -crotonyl CoA reductase (110, 157), $NADH_2$, and $NADPH_2$, synthesized octanoic and decanoic acids from hexanoyl CoA and C^{14} -acetyl CoA.

Preliminary observations (203, 208) regarding the possible involvement of pyridoxal phosphate in the mitochondrial elongation of fatty acids suggest the presence of a new type of condensation reaction sufficiently different from the thiolase reaction as to alter specificity and to overcome the unfavorable equilibrium of the latter enzyme. The partial requirement for pyridoxal phosphate in the elongation of fatty acids by charcoal-treated soluble preparations from mitochondria was reported (203, 208). Although an absolute requirement was not demonstrable, twofold stimulation was observed. This may be considered as an initial step toward confirmation of the results of many nutritional studies (16, 31, 42, 71, 161, 184, 215), namely, that a direct relationship exists between vitamin B_6 requirement and the metabolism of essential fatty acids.

The nonmitochondrial system for fatty acid synthesis.—Gurin and his co-workers (25, 50, 196) first reported the synthesis of long-chain fatty acids from acetate catalyzed by soluble extracts of pigeon liver. Treatment of such extracts with charcoal resulted in a loss of enzymatic activity which could be partially restored by the addition of ATP, NAD, and CoA; citrate and Mg^{++} were stimulatory. A system isolated by Popják & Tietz (143, 191) from mammary gland had the same general characteristics as did that from pigeon liver. Wakil *et al.* (63, 64, 145, 146, 202, 206, 209, 210) determined the cofactor requirement for maximal conversion of acetate to fatty acids and separated the extracts into two distinct fractions which were several-fold purified. They showed that this system was located primarily in the supernatant fluid obtained after centrifugation in 0.25M or 0.85M sucrose at 100,000 g for 1 hr. Addition of microsomes or mitochondria did not increase the conversion of acetate to fatty acids. However, centrifugation of the clear supernatant solution at 140,000 g for 2 to 4 hr yielded red pellets which contained the fatty acid-synthesizing enzymes.

The soluble pigeon liver extracts were fractionated by conventional

methods to two distinct fractions which, on combination, could convert acetyl CoA to palmitate. Bicarbonate, ATP, Mn^{++} , and $NADPH_2$ were absolutely required for the synthesis of fatty acids from acetyl CoA by the purified enzymes. The cofactors required for fatty acid synthesis by this system were different from those to be expected from a reversal of β -oxidation. C^{14} -labeled CO_2 did not incorporate into the fatty acid (63, 64, 210), hence a catalytic role for this component was postulated. The bicarbonate requirement was appreciated only after studies on the purified enzymes; earlier reports of the stimulatory effects of bicarbonate buffers and gaseous CO_2 on fatty acid synthesis, respectively, in rat liver slices (23) and cell-free extracts from yeast (96) had remained obscure.

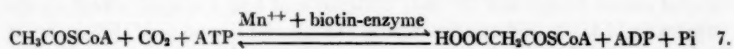
Using the criteria of CO_2 requirement, the universal distribution of this pathway in living organisms could be shown (3, 20, 25, 48, 57, 62, 103, 104, 116, 126, 143, 168, 182, 189, 203, 209). Klein (96) studied fatty acid synthesis in yeast extracts and observed a requirement for a small particle fraction which sedimented between 25,000 and 60,000 g (free from the cytochromes and cytochrome oxidase) in addition to the soluble supernatant. These particles are not derived from mitochondria, and recently Klein (97) was able to show that they bear no direct relationship to yeast ribonucleoprotein particles, since full activity was retained after hydrolytic removal of 80 per cent of the ribonucleic acid by ribonuclease. As will be discussed later, Den & Klein (45) presented evidence to show that these particles catalyzed the carboxylation of acetyl CoA to malonyl CoA in the presence of ATP and Mn^{++} . Matthes *et al.* (1, 5, 128) reported that soluble extracts of rat liver required microsomes for the synthesis of fatty acids. The addition of microsomes to the supernatant fraction caused a five- to tenfold stimulation of lipogenesis from acetate, whereas earlier workers could not find such stimulation (25, 50, 110, 143, 191, 196, 203, 209).

Stumpf and his co-workers (7, 136, 182) studied fatty acid synthesis in cell-free extracts of avocado mesocarp. Although this plant system includes acetyl CoA carboxylase and can convert malonyl CoA to long-chain fatty acids, it appears to be completely associated with mitochondrial particles and to yield esterified (triglyceride and phospholipids) fatty acids with different chain lengths from those found in the animal systems. Further information is needed in order to resolve this apparent contradiction. Information pertinent to the cellular distribution of acetyl CoA carboxylase and the enzymes that convert acetyl CoA and malonyl CoA to palmitate will be a welcome addition to our knowledge of the system and may shed new light on the two claims.

Product of synthesis.—Brady & Gurin (25) showed that soluble pigeon liver extracts could catalyze the *de novo* synthesis of long-chain fatty acids ($>C_{10}$) from C^{14} -acetate. Similar results were reported by Popják & Tietz (143) using mammary gland homogenates which converted acetate mainly to fatty acids of chain lengths C_{10} to C_{18} . These observations were also confirmed by Porter & Tietz (145), who showed palmitic acid to be the major component synthesized (60 to 80 per cent) by purified preparations from

pigeon liver with stearic and myristic acids constituting the remainder. Furthermore, no short-chain fatty acids (C_4 to C_{10}) accumulated during synthesis. It is of interest to note here that the C_{18} acids (palmitic and palmitoleic acids) are the most abundant acids in nature. The fatty acids synthesized by this system were shown to be the free fatty acids (28) and not the CoA derivatives as reported earlier by Porter & Long (144). Recently, Mudd & Stumpf (136) reported that palmitate, stearate, and oleate were the main fatty acids synthesized by the particulate system of avocado mesocarp, whereas only the saturated acids were synthesized by the acetone-powder extracts of these particles (7).

Acetyl CoA carboxylase.—Recently, Wakil (202) isolated the first intermediate in the synthesis of long-chain fatty acids from acetyl CoA. This intermediate, identified as malonyl CoA, was formed by the carboxylation of acetyl CoA in the presence of ATP, Mn^{++} , and a highly purified enzyme fraction derived from the pigeon liver extracts. The stoichiometry of the reaction has been shown to involve the effective hydrolysis of ATP to ADP and inorganic phosphate as shown in the following reaction:



The enzyme has been called acetyl CoA carboxylase (203) and the reaction appears to be reversible (212).

In 1958, Wakil *et al.* (64, 207, 210) reported the high biotin content of this enzyme and demonstrated the participation of biotin in the synthesis of fatty acid by the use of avidin as a specific inhibitor. The biotin-binding site of avidin appears to be responsible for this inhibition, since biotin-saturated avidin is unable to inhibit malonyl CoA formation. Since the first isolation of the biotin-enzyme in fatty acid synthesis, the use of the avidin inhibition technique has revealed several new biotin enzymes (74, 90, 116, 117, 185).

Acetyl CoA carboxylase has been purified extensively from chicken liver extracts, and at the highest purity level contained 1 mole of biotin per 500,000 to 750,000 g of protein. The biotin is covalently bound to the enzyme and can be released only by acid hydrolysis or by digestion with various peptidases (207, 212).

The carboxylase is specific for Mn^{++} ; ATP can be replaced by uridine triphosphate, but only at higher concentration. Propionyl CoA can also be carboxylated by acetyl CoA carboxylase, but at about one-third to one-fourth the rate of acetyl CoA carboxylation. The product of propionyl CoA carboxylation has been shown to be methylmalonyl CoA (212).

The presence of a similar enzyme in pig heart extracts was reported by Formica & Brady (54), and recently Den & Klein (45) isolated a particle from yeast cells (*Saccharomyces cerevisiae*) which carboxylates acetyl CoA to form malonyl CoA in the presence of ATP and Mn^{++} .

Effect of isocitrate on acetyl CoA carboxylase.—The general interest in the effect of di- and tricarboxylic acids on the *in vitro* synthesis of fatty acids

stems from the original observations of Brady & Gurin (25) that citric acid markedly stimulated fatty acid synthesis in extracts of pigeon liver. Popják & Tietz (143, 191) observed similar effects in extracts of mammary gland and extended the list of acids to include malonate, α -ketoglutarate, and oxalacetate. Similar observations were reported also by Porter *et al.* (146), and more recently by Chaikoff and his co-workers (1, 5, 128). Porter *et al.* (146) found that isocitrate gave the highest stimulation of fatty acid synthesis in a partially purified enzyme system, whereas malonate was half as effective as isocitrate.

A possible explanation for the effect of malonate was thought to involve the activation of malonate to malonyl CoA (21) which was then utilized for fatty acid synthesis. Dils & Popják (48) pointed out that this could not be the case because, had malonate been converted to malonyl CoA, a depression of acetate conversion to fatty acids caused by dilution effect would have been expected rather than the observed stimulation. Dils & Popják (48) presented two explanations for this phenomenon: (a) that malonic acid prevented the deacylation of malonyl CoA (formed by the carboxylation of labeled acetyl CoA); and (b) that malonic acid had a sparing effect on the substrates of fatty acid synthesis (acetyl CoA, ATP, and NADPH₂), for, at high concentrations of these substrates, malonic acid was without effect on fatty acid synthesis.

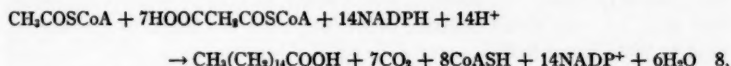
Waite (199) purified acetyl CoA carboxylase about 500-fold over crude chicken liver extracts and found that the addition of isocitrate to the reaction mixture containing acetyl CoA, ATP, CO₂, and Mn⁺⁺ resulted in a sixfold increase in the rate of synthesis of malonyl CoA. Other di- or tricarboxylic acids (citrate, α -ketoglutarate, succinate, oxalacetate, malonate, malate, and itaconate) caused much less increase in malonyl CoA formation (two- to threefold) than did isocitrate. It is significant that the concentrations of the di- and tricarboxylic acids were very high ($10^{-3}M$) compared to the various components of the reaction. These phenomena could also be demonstrated at various levels of reactants, whether varied individually or collectively. These observations do not lend support to the aforementioned explanation of Dils & Popják (48). Moreover, the earlier explanation (1, 5, 63) that isocitrate stimulates because it is a source of both CO₂ and NADPH₂ is also inadequate, since the stimulatory effect was noted when the carboxylase reaction alone was studied in the presence of maximum concentration of HCO₃⁻. Further information appears to be necessary in order to understand fully this stimulation of malonyl CoA formation by isocitrate and other acids.

Formation of malonyl CoA by reactions other than the carboxylation of acetyl CoA.—Several biosynthetic routes to malonyl CoA have been shown to be operative, in addition to the aforementioned carboxylation of acetyl CoA. They are: (a) activation of malonic acid in the presence of ATP and CoA by a specific thiokinase first described by Hayaishi in *Pseudomonas fluorescens* (75) and by Nakada *et al.* in rat kidney (138); (b) the CoA-

transferase reaction in which CoA is exchanged between succinyl CoA and malonate and between acetoacetyl CoA and malonate, as recently described by Menon & Stern (134); (c) the oxidation of malonyl semi-aldehyde CoA to malonyl CoA in *Clostridium kluyveri* (193).

Conversion of malonyl CoA to palmitic acid.—Identification and biosynthesis of malonyl CoA as the first intermediate in fatty acid synthesis was a key step towards understanding fatty acid biosynthesis. Malonyl CoA was readily converted to fatty acids in the presence of the second enzyme fraction (202, 206). Wakil & Ganguly (206) found that acetyl CoA was essential for this conversion; it provided the building block or "primer" onto which the "C₂ units" derived from malonyl CoA are attached. CO₂ is released during this condensation and NADPH₂ provides the electrons needed for the complete reduction of the carbonyl to the methylene groups. NADH₂ could substitute for NADPH₂ in fatty acid synthesis, but the rate of oxidation of NADH₂ is about one-third the rate of oxidation of NADPH₂ (22, 116, 211). Similar observations were later reported in preparations from rat liver (22, 57, 62) brain (20), adipose tissue (126), mammary gland (3, 48), yeast cells (116), and avocado mesocarps (7, 136).

The stoichiometric relationship (22, 27, 126, 203) between the reactants (acetyl CoA, malonyl CoA, and NADPH₂) and the products (palmitic acid, CO₂, CoA, and NADPH₂) is illustrated in reaction 8.



Acetyl CoA is incorporated into the tail end of the fatty acid (carbon 15 and 16) (22, 27, 203, 206), whereas malonyl CoA provides the C₂ units for condensation and therefore carbon atoms 1 to 14.

Bressler & Wakil (27) demonstrated the incorporation of tritium from tritium labeled acetyl CoA (CT₃COSCoA) or tritium labeled malonyl CoA (HOOCCT₂COSCoA) into palmitic acid in the presence of NADPH₂ and the purified enzyme fraction from pigeon liver. One mole of tritium labeled acetyl CoA (equivalent to the intact methyl group) was incorporated per mole of palmitic acid. This is in conformity with the stoichiometry of the reaction as expressed in reaction 8. When tritium labeled malonyl CoA was used in the presence of nonlabeled acetyl CoA, tritium was found in the palmitic acid to the extent of five to seven atoms of tritium per mole of palmitic acid, or one atom of tritium per mole of malonyl CoA converted to palmitic acid. These results confirmed the earlier findings of Sonderhoff & Thomas (167) that deuterated acetic acid (CD₃COOH) could be incorporated into long chain fatty acid by yeast cells. It also confirms Rittenberg & Bloch's (149, 150) results on the incorporation by mice and rats of deuterium labeled acetate into long chain fatty acids.

The incorporation of tritium from NADPH₂ into palmitic acid was reported by Brady *et al.* (22) in purified preparations from rat liver extracts.

Stepwise degradation of palmitic acid to dodecanoic acid was performed, and the hydrogen atoms of carbons 2, 3, 4, and 5 were trapped as H_2O . The results indicated that the hydrogens of carbons 3 and 5 contained significant amount of tritium as compared to the hydrogens of carbons 2 and 4. Thus, tritium from $NADPH_2$ is localized on alternate carbon atoms of palmitic acid, beginning with carbon atom 3. These results are consistent with the concept of direct transfer of hydrogen atoms from reduced pyridine nucleotides to carbon 3 of β -ketoacyl CoA reported earlier by Marcus *et al.* (123). Because of chemical and enzymatic considerations, and in analogy to fatty acid oxidation, it is reasonable to assume that there may be three distinct steps involved in the complete reduction of the carbonyl group ($>C=O$) to the methylene group ($>CH_2$) (206): the reduction of carbon-oxygen bond, the elimination of water molecule, and in the third step the reduction of the carbon-carbon bond ($>C=C<$). The results of Brady *et al.* (22) did not indicate in which step the direct transfer occurred. Recently, Lynen & Tada (120) reported that flavin mononucleotide is a cofactor in the reduction of α - β -unsaturated acyl CoA by $NADPH_2$ in the presence of purified enzyme preparations from yeast extracts. Since they considered this to be one of the reductive steps involved in fatty acid synthesis, flavin mononucleotide presumably intercepts electrons from $NADPH_2$ and transfers them to the α - β -unsaturated acyl intermediate in fatty acid synthesis. If this is the case, then loss of tritium to the medium would occur, leaving only the other reductive step, i.e., the direct transfer of tritium from $NADPH_2$ to the β -ketoacyl to yield the β -hydroxyacyl derivative. More rigorous proof is needed in order to ascertain this particular point. In a preliminary article, Abraham *et al.* (4) reported that tritium from the α -form NADPH was transferred to the fatty acids more readily than from the β -form NADPH.

Requirement for acyl CoA in the conversion of malonyl CoA to fatty acids.—Acetyl CoA is absolutely required for the conversion of malonyl CoA to palmitic acid. Other acyl CoA's (propionyl CoA, butyryl CoA, isobutyryl CoA, isocaproyl CoA, isovaleryl CoA, and α -methylbutyryl CoA) (20, 80, 206) can condense with malonyl CoA to form various long-chain fatty acids, depending upon the nature of the primer acyl CoA employed. The extent to which these various acyl CoA derivatives promote fatty acid synthesis from malonyl CoA is questionable and controversial. Wakil & Ganguly (206) reported in 1959 the participation of acetyl CoA, butyryl CoA, and octanoyl CoA in the conversion of malonyl CoA to fatty acids, and on the inability of substituted acyl derivatives of CoA (β -hydroxybutyryl CoA, crotonyl CoA, and acetoacetyl CoA) to oxidize $NADPH_2$ or to incorporate into long-chain fatty acids. A scheme for palmityl CoA synthesis was proposed which involved dicarboxylic acyl CoA derivatives as possible intermediates.

More recent studies by Bressler & Wakil (27, 203) on the mechanism of fatty acid synthesis proved that the aforementioned hypothesis was untenable. They found that propionyl CoA can partially substitute for acetyl CoA, whereas butyryl CoA can do so only to a much lesser extent. This was reflected in the Michaelis-Menten constants: acetyl CoA, $2.3 \times 10^{-6} M$;

propionyl CoA, $5.7 \times 10^{-5} M$; and butyryl CoA, $3.9 \times 10^{-4} M$. Experiments (including trapping and dilution techniques) designed to isolate short-chain acyl CoAs (butyryl CoA, hexanoyl CoA) from the reaction mixture during the synthesis of long-chain fatty acids from C^{14} -labeled malonyl CoA were not successful. To date, isolation of short-chain acyl CoA compounds from other systems where such compounds (butyryl CoA, hexanoyl CoA, etc.) are assumed to be intermediates has not been reported (20, 80, 120, 127). It is of interest to note that in 1950 Brady & Gurin (23), using rat liver slices, showed that carboxyl- C^{14} butyrate, hexanoate, and octanoate were not incorporated into long-chain fatty acids as intact units. Their results indicated that these compounds were probably cleaved to acetate which was subsequently converted to long-chain fatty acids. The inability of short-chain acyl CoA derivatives to incorporate into higher fatty acids by the purified enzyme system (27) substantiates Brady & Gurin's results.

Horning *et al.* (80) studied the synthesis of odd-numbered and branched-chain fatty acids in enzyme preparations from rat adipose tissue. The type of fatty acid synthesized was shown to depend upon the nature of the acyl CoA primer. Thus, when propionyl CoA was used as primer, odd-chain fatty acids were isolated. The purified enzymes from rat-adipose tissue yielded mainly pentadecanoic acid (195) from propionyl CoA, whereas the purified enzymes from pigeon liver produced mainly heptadecanoic acid (27). Using isobutyryl CoA, isocaproyl CoA, isovaleryl CoA, or α -methylbutyryl CoA as a primer in the purified enzyme system from adipose tissues, Vagelos *et al.* (195) isolated as major products iso- C_{16} , iso- C_{18} , plus iso- C_{17} , or anteiso- C_{18} plus anteiso- C_{17} , fatty acids, respectively.

Substituted acyl CoA derivatives such as β -hydroxybutyryl CoA and crotonyl CoA did not substitute for acetyl CoA in the conversion of malonyl CoA to palmitate; neither did they oxidize $NADPH_2$ in the presence of the purified pigeon liver enzyme system (27, 206). Similar observations were reported on the inability of substituted fatty acyl CoA's to oxidize $NADPH_2$ and to incorporate into long-chain fatty acids in presence of purified preparations from yeast cells (120), rat brain (20), rat liver (22), and adipose tissue (126). Acetoacetyl CoA appears to behave differently from crotonyl CoA and β -hydroxybutyryl CoA. When acetoacetyl CoA was substituted for acetyl CoA in a reaction mixture containing C^{14} -malonyl CoA ($HOOCCH_2C^{14}OSCoA$), $NADPH_2$, and the purified enzyme fraction from pigeon liver, there was a significant amount of incorporation of C^{14} into fatty acids, as well as an increase in the rate of oxidation of $NADPH_2$ (205). The rate of incorporation of C^{14} into fatty acids and the oxidation of $NADPH_2$ were less than that obtained with acetyl CoA. Higher concentrations of acetoacetyl CoA were required in order to approach rates obtained with acetyl CoA. The data (205) did not permit a decision as to whether promotion of fatty acid synthesis in the presence of acetoacetyl CoA was caused by its condensation with malonyl CoA as in intact C_4 unit, its possible cleavage to acetyl CoA, or to both.

When acetoacetyl CoA and $NADPH_2$ were incubated with the fatty acid

synthesizing system, there was a rapid oxidation of NADPH_2 (120, 205). The enzyme that catalyzes the reduction of acetoacetyl CoA by NADPH_2 was named acetoacetyl CoA reductase because of the reductive nature of the reaction. The product of reduction of acetoacetyl CoA was isolated from the reaction mixture and was identified enzymatically as $\text{D}(-)\beta$ -hydroxybutyryl CoA (205). When the reduction of acetoacetyl CoA by NADPH_2 was catalyzed by a protein fraction containing all of the enzymes required for the synthesis of palmitic acid, the product was always found to be $\text{D}(-)\beta$ -hydroxybutyryl CoA regardless of the length of incubation or the concentration of NADPH_2 . This would indicate that this product is not reduced further by NADPH_2 to butyryl CoA as would have been expected if this reaction were part of the series of reactions of fatty acid synthesis. Nonlabeled $\text{D}(-)\beta$ -hydroxybutyryl CoA did not trap C^{14} from labeled malonyl CoA ($\text{HOOCCH}_2\text{C}^{14}\text{OSCoA}$) during active synthesis of fatty acids. These observations do not support the concept that free $\text{D}(-)\beta$ -hydroxybutyryl CoA is an intermediate (or in equilibrium with intermediates) in fatty acid synthesis (205).

Further evidence was obtained which indicated that the acetoacetyl CoA reductase was not part of the fatty acid-synthesizing enzymes. The ratio of the two activities varied throughout the purification steps, from about 4:1 in favor of the reductase in crude extracts to 1:20 in favor of the fatty acid synthesis at the highly purified stage (27). Acetoacetyl CoA reductase cannot be inhibited by SH-binding reagents such as *p*-hydroxymercuribenzoate or *N*-ethylmaleimide in concentrations up to $10^{-3}M$, whereas the fatty acid-synthesizing system was completely inhibited by these reagents at concentrations of about $10^{-5}M$ (205). Thus, in the presence of SH-inhibitors it was possible to obtain preparations that retained the full reductase activity but were devoid of fatty acid synthetic activity.

The physiological role of the acetoacetyl CoA reductase is not known as yet. This enzyme may not be involved in the over-all synthesis of palmitic acid from acetyl CoA and malonyl CoA, and a role in ketone body formation is proposed by Wakil & Bressler (205). On the other hand, Lynen & Tada (120) included this enzyme in their proposed scheme for fatty-acid synthesis in yeast preparations.

Condensation of acetyl CoA and malonyl CoA.—The stoichiometry of fatty acid synthesis (reaction 8) shows that malonyl CoA is decarboxylated during the over-all reaction and that the carboxyl carbon of carboxyl-labeled malonyl CoA ($\text{HOOC}^{14}\text{CH}_2\text{OSCoA}$) did not incorporate into the fatty acids (27). This observation is in conformity with the original hypothesis (63, 64) that the role of CO_2 is catalytic in nature. Attempts by various workers (22, 126, 206) to demonstrate an acetyl CoA-dependent decarboxylation of malonyl CoA in the absence of NADPH_2 were unsuccessful because of the presence of a contaminating enzyme which decarboxylated malonyl CoA to acetyl CoA plus CO_2 as shown in reaction 9.



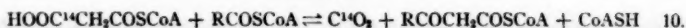
Such a decarboxylase has been studied by Hayaishi in *Pseudomonas fluorescens* (75) and by Nakada *et al.* (138) in rat liver mitochondria.

In the absence of NADPH_2 , Bressler & Wakil (27) were able to demonstrate the acetyl CoA-dependent decarboxylation of malonyl CoA by the purified fatty acid-synthesizing system. The liberation of C^{14}O_2 from carboxyl-labeled malonyl CoA ($\text{HOOC}^{14}\text{CH}_2\text{COSCoA}$) was dependent upon the presence of acetyl CoA and was proportional to both time and enzyme concentration. The rate of CO_2 release followed very closely the over-all rate of fatty acid synthesis. Acetyl CoA would be replaced by propionyl CoA and, to a lesser extent, by butyryl CoA. At optimal concentrations, propionyl CoA caused the release of 40 per cent as much C^{14}O_2 from malonyl CoA as did acetyl CoA, whereas butyryl CoA caused the release of only 4 per cent as much C^{14}O_2 as did acetyl CoA (27). These results are in line with the observed behavior of these esters of CoA in their substitution for acetyl CoA in the over-all synthesis of fatty acids.

Product of condensation of acetyl CoA and malonyl CoA.—As a result of the condensation of acetyl CoA and malonyl CoA in the absence of NADPH_2 , there was an accumulation of a compound, in addition to the release of CO_2 (27). The addition of NADPH_2 to a reaction mixture containing acetyl CoA and malonyl CoA completely prevented the accumulation of the product of the decarboxylation reaction, even under conditions where excess of substrates (acetyl CoA and malonyl CoA) was present. This product was extracted from the reaction mixture and was readily separated from acetic, malonic, crotonic, acetoacetic, β -hydroxybutyric, and butyric acids by paper chromatography (27). C^{14} -Carbonyl labeled malonyl CoA ($\text{HOOCCH}_2^{14}\text{OSCoA}$), 2- C^{14} -labeled malonyl CoA, as well as C^{14} -labeled acetyl CoA, were incorporated into this compound, whereas C^{14} -carboxyl-labeled malonyl CoA yielded C^{14}O_2 . Propionyl CoA and butyryl CoA could substitute for acetyl CoA, but to a lesser extent, and were incorporated into the condensation product with the simultaneous release of CO_2 . The extent of incorporation of C^{14} -propionyl CoA and C^{14} -butyryl CoA, *vis à vis* acetyl CoA, is similar to their relative incorporation into fatty acids in the over-all system.

Spectrophotometric examination of the isolated compound revealed an absorption maximum at about 275 $\text{m}\mu$ (28). The formation of this compound can be followed directly in a reaction containing acetyl CoA, malonyl CoA, and the fatty acid-synthesizing enzymes by measuring the increase in optical density at 275 $\text{m}\mu$. A simultaneous decrease in optical density at 235 $\text{m}\mu$ was also noted (28). This was caused by the disappearance of the thioester linkage of acetyl CoA as it is consumed in the condensation reaction. The exact nature of this compound and its relation to fatty acid synthesis is not yet understood. It has not been possible to convert it to palmitate.

Malonyl CoA- CO_2 exchange reactions.—Vagelos & Alberts (194) demonstrated in extracts of *Clostridium kluyveri* an exchange reaction between malonyl CoA and C^{14}O_2 that is completely dependent upon the addition of an acyl CoA. The exchange reaction can be presented as follows:



The enzyme has been purified about 75-fold, but the product of the reaction has not been identified because of interfering enzymes. The highest exchange values can be obtained with caproyl CoA (C_6CoA) and higher acyl CoA derivatives. Acetyl CoA and butyryl CoA are not as active as the longer-chain homologue of acyl CoA derivatives (194).

Martin *et al.* (126) were also able to demonstrate, in crude enzyme extracts from adipose tissue, a similar exchange reaction of C^{14}O_2 with malonyl CoA in the presence of a fatty acyl CoA such as caproyl CoA. The total activity of the enzyme in such extracts was very low as compared to extracts of *Cl. kluyveri*, though the requirements of the reaction were the same. A similar exchange reaction was also reported by Lynen & Tada (120) in the purified enzyme preparation from yeast cells. Martin *et al.* (126), as well as Lynen & Tada (120), have used this exchange reaction as evidence for the condensation-decarboxylation reaction. Direct evidence has been presented to demonstrate acetyl CoA dependent decarboxylation of malonyl CoA, with relatively little or no exchange between C^{14}O_2 and malonyl CoA in the presence of an acyl CoA (27, 81). The fact that caproyl CoA cannot replace acetyl CoA for fatty acid synthesis (27, 127), together with the findings of Martin *et al.* (126) that acetyl CoA was less active than caproyl CoA in promoting the exchange between C^{14}O_2 and malonyl CoA, makes it unlikely that the exchange reaction is part of the over-all synthetic scheme for fatty acids.

More recently, Goldman *et al.* (70) were able to obtain three separate protein fractions from crude extracts of *Cl. kluyveri* which on combination could catalyze the synthesis of long-chain fatty acids from malonyl CoA in the presence of NADPH_2 . Since a combination of two of these fractions catalyzed the C^{14}O_2 -malonyl CoA exchange reaction, it was taken by the authors to imply a role for the exchange reaction in the over-all synthesis of fatty acids. Further information is necessary in order to establish the unequivocal involvement of the exchange reaction in the enzymatic sequence of fatty acid synthesis and to ascertain the relationship between the exchange reaction and the acetyl CoA dependent decarboxylation of malonyl CoA (27).

Role of SH groups in fatty acid synthesis.—The role of sulfhydryl groups in the synthesis of fatty acid was first described by Wakil and co-workers, working with preparations from avian liver (146, 209). Other workers reported similar observations in extracts of yeast (120), brain (20), and adipose tissue (80). Sulfhydryl reagents such as glutathione, cysteine, and mercaptoethanol stimulate fatty acid synthesis, whereas sulfhydryl binding agents such as *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, cadmium ions, and arsenite inhibit the synthesis. Over 85 per cent inhibition of fatty acid synthesis was obtained in purified preparations from pigeon liver when the concen-

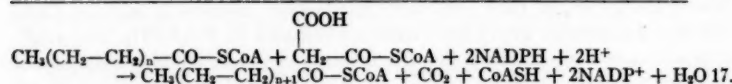
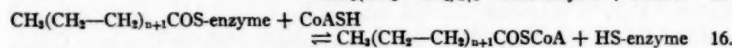
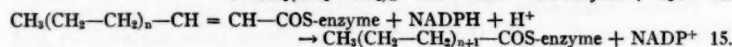
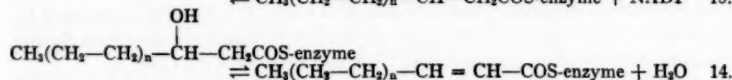
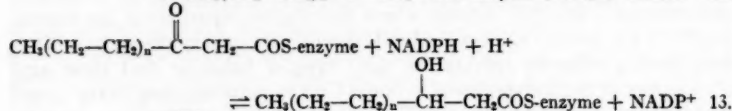
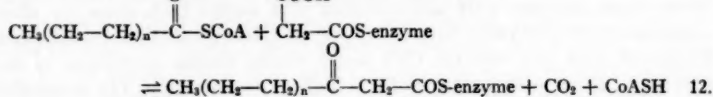
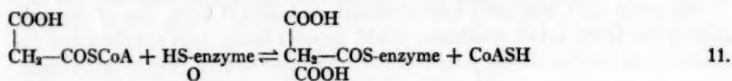
tration of *p*-hydroxymercuribenzoate was $2.5 \times 10^{-2} M$ (28). Similar inhibition of acetyl CoA dependent carboxylation of malonyl CoA was obtained at about the same concentration of *p*-hydroxymercuribenzoate (28).

Recently (28), evidence was obtained that acetyl CoA, one of the substrates for fatty acids synthesis, could protect fatty acid-synthesizing enzymes from inhibition by *p*-hydroxymercuribenzoate or *N*-ethylmaleimide. When these enzymes were preincubated with acetyl CoA, prior to their exposure to the sulfhydryl binding agents, complete protection was afforded. Propionyl CoA and butyryl CoA could render similar protection of the enzyme, but to a lesser extent and at a higher concentration. The protection phenomenon is another instance where the relative capacities of the various acyl CoA's to protect against the inhibition of fatty acid synthesis by sulfhydryl binding reagents reflects the same type of behavior that these acyl CoA's show in the conversion of malonyl CoA to palmitate and in the acetyl CoA dependent decarboxylation of malonyl CoA (27). Similar protection by these acyl CoA's was also found against the inhibition of the decarboxylation condensation reaction between acyl CoA and malonyl CoA (28).

Possible mechanism of fatty acid synthesis.—The earlier suggestion by Wakil & Ganguly (206) that fatty acid synthesis from acetyl CoA and malonyl CoA is achieved via the intermediary formation of substituted malonyl CoA's, was based upon the then available evidence that acyl CoA's (acetyl CoA, butyryl CoA, etc.) were condensing partners of malonyl CoA, and that substituted acyl CoA's were not reduced by NADPH₂. The availability of newer information, i.e., the inability of longer-chain homologues of acyl CoA's to substitute for acetyl CoA in the over-all conversion of malonyl CoA to palmitic acid, the demonstration that acetyl CoA dependent decarboxylation of malonyl CoA in the absence of NADPH₂, and the incorporation into plamitate of one atom of tritium per mole of malonyl CoA, made the dicarboxylic acid hypothesis of fatty acid synthesis untenable (27, 201, 203). At least, two hypotheses are now available to explain the synthesis of long-chain fatty acid from malonyl CoA, acetyl CoA, and NADPH₂ (120, 205).

The inhibition of the fatty acid-synthesizing enzymes by SH-binding agents, as discussed previously, and the ability of these enzymes to bind acyl CoA's (22, 27, 28, 120) has led Lynen & Tada to propose the formation of tightly bound acyl-S-enzyme complexes as intermediates in the conversion of acetyl CoA and malonyl CoA to palmitate. This hypothesis is similar to an early mechanism proposed by Lardy for the β -oxidation of fatty acids by fatty acid oxidase (111). The proposed scheme of synthesis assumes a reaction between malonyl CoA and enzyme-SH to form malonyl-S-enzyme complex, which then condenses with an acyl CoA (acetyl CoA, butyryl CoA, hexanoyl CoA, etc.). This is then reduced by NADPH₂, dehydrated and again reduced by NADPH₂ via flavin mononucleotide to form a saturated acyl-S-enzyme. The complex then reacts with CoASH to form an acyl CoA

and enzyme-SH. The scheme assumes the presence of one enzyme complex responsible for the entire sequence of reactions leading to the synthesis of long-chain fatty acids as shown by the following reactions:

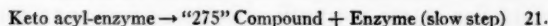
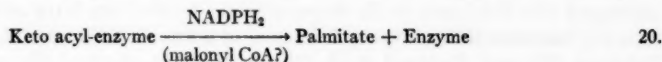
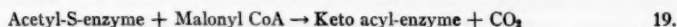
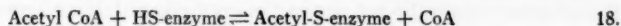


This hypothesis was based on results obtained from studies on the fatty acid-synthesizing system derived from yeast extracts (120). A 200-fold purified enzyme fraction with an estimated molecular weight of 2,000,000 was obtained from yeast extract which behaved as one component on examination in the ultracentrifuge and Tiseleus electrophoresis. The enzyme preparation catalyzed the conversion of acetyl CoA and malonyl CoA to palmityl CoA and stearyl CoA in the presence of NADPH₂. Acetyl CoA could be readily substituted by butyryl CoA, hexanoyl CoA, and myristyl CoA. The rate of incorporation of the longer-chain acyl CoA into the final products was much higher than acetyl CoA, indicating that these acyl CoA's are intermediates in the pathway of synthesis of palmityl CoA and stearyl CoA. A molecule of the enzyme is proposed to interact with a molecule each of malonyl CoA and acetyl CoA to form an acetoacetyl-S-enzyme complex. The proposed enzyme complex contained six different activities arranged around a sulfhydryl axis in such a way as to make available the acyl group to the active center of each protein. Detailed experimental evidence to support this has not been published as yet and it is very difficult, therefore, to consider seriously its applicability to the yeast system. The steric problems between the various enzymatic loci of such a complex may be enough to discourage its formation; nor is there precedent for the formation of a complex of this type.

The closest multi-enzyme complex is the one involved in the oxidation of pyruvate or α -ketoglutarate. In these complexes, however, there are several prosthetic groups involved where the substrate or its degradative product is bound during its transformation with simultaneous oxidation-reduction of the coenzymes (147). No such transacylation of the substrates is postulated by Lynen & Tada, though six different enzymatic reactions are involved.

The recent results of Wakil and co-workers (27, 201, 203) on the synthesis of palmitic acid from malonyl CoA and acetyl CoA do not lend support to Lynen's hypothesis. Whether this variance results from the different sources of the enzyme systems used remains to be clarified.

Any interpretation of the available data must postulate either the presence of a new cofactor (including an acyl-S-enzyme) that may be involved in the formation of the active intermediates, or the possible formation of a polyketo derivative which can be reduced, possibly stepwise, by NADPH₂ to the fully saturated fatty acids. The "275" compound isolated by Bressler & Wakil (28) may be such a keto acid, or closely related to it, and the heat-stable cofactor that was reported recently by Goldman *et al.* (70), which was required for the C¹⁴O₂-malonyl CoA exchange reaction from *Cl. kluyveri*, may be the new conjugated partner for the formation of the various intermediates in fatty acids and their ultimate conversion to palmitate. Schematically, therefore, fatty acid synthesis can be presented as follows:



The enzyme may be substituted by the cofactor of Goldman *et al.* and malonyl CoA may or may not be required in the final conversion of the active "275" compound to palmitate. The complete reduction of the carbonyl to methylene groups ($>\text{C}=\text{O} \rightarrow >\text{CH}_2$) by NADPH₂ would most probably be accomplished in three stages: first, the reduction of the carbonyl group to the secondary alcohol, then the removal of water, and finally the reduction of the carbon-carbon double bond (most probably via a flavin nucleotide) to the saturated carbons. It is obvious that further information is necessary to ascertain which of these mechanisms or alternatives is operative.

Effect of starvation and diabetes on fatty acid synthesis.—Evidence accumulated during the past two decades indicates that fatty acid synthesis is significantly reduced in diabetic or starved animals (24, 33, 34, 51, 160, 178). Administration of insulin to the diabetic animal or refeeding of starved animals completely restores the ability of these animals to synthesize fatty acids. The failure of diabetic or starved animals to synthesize fatty acids has been attributed to many factors, including the effect of glucose metabolism

on fatty acid synthesis and the levels of the enzyme system required for the synthesis of fatty acids (24, 33, 164, 169). Alteration of glucose metabolism is bound to influence fatty acid synthesis, since glucose is the main source of both the carbon atoms and the reducing power necessary for the synthesis of fatty acids. These and perhaps other unknown changes may also be reflected in adaptive changes in the specific activity and yield of several enzyme systems (among them is the fatty acid-synthesizing enzyme system) (61, 66).

The recent finding that NADPH_2 is the electron donor in fatty acid synthesis prompted the suggestion that the decrease in NADPH_2 generation resulting from the diminished specific activity of the pentose cycle dehydrogenases might be the defective step in lipogenesis in the livers of diabetic or starved animals (110, 162, 163, 164, 170). Further support for this hypothesis was obtained by Tepperman & Tepperman (188) who found that fatty acid synthesis as well as glucose-6-phosphate dehydrogenase levels were substantially increased in liver slices from rats refed a high carbohydrate and low-fat diet. More recently, Abraham *et al.* (2, 128) and Gibson and his co-workers (28, 61, 81) have obtained evidence to show that the activity of the fatty acid-synthesizing system in liver preparations from diabetic or starved animals is one-tenth to one-third that observed in preparations from normal animals. Addition of the NADPH_2 or NADPH_2 -generating systems to such preparations did not restore the synthetic activity, whereas pretreatment of the diabetic animals with insulin or refeeding of the starved animals completely restored the fatty acid synthesizing activity. Both authors attributed this deficiency to the decrease in the level of the fatty acid-synthesizing enzymes rather than a deficiency of a known cofactor. Gibson & Hubbard (62) and Hubbard *et al.* (81) studied the effect of diabetes or starvation on the enzymatic activities of both acetyl CoA carboxylase and the enzymes that catalyze the conversion of malonyl CoA to palmitate in order to define more precisely the site of defect in the over-all synthesis of fatty acids. Using direct enzymatic assay, these workers found that acetyl CoA carboxylase activity was not impaired in diabetes or starvation where the enzyme system responsible for the conversion of malonyl CoA to palmitate is significantly reduced. Furthermore, refeeding of starved animals with a fat-free diet significantly increased (10 times above the level of extracts from starved animals or two to four times that from normal animals) the ability of the extracts to convert malonyl CoA to palmitate. Similar results were obtained in our laboratory (28, 205) and this technique of starving animals for 48 hr and refeeding them 24 hr prior to extracting their livers is used routinely to obtain higher activities of these enzymes.

Numa *et al.* (140) reported on similar studies in preparation from livers of fasted and alloxan diabetic rats and found that the level of all enzymes involved in fatty acid synthesis via malonyl CoA was lower in these animals than in normal ones. They also found that the rate limiting step in fatty acid synthesis in both experimental and control animals was the carboxylation of

acetyl CoA. Addition, therefore, of purified acetyl CoA carboxylase to various extracts increased fatty acid synthesis, and in this way they were able to restore and even surpass the synthesis in extracts from fasted animals to the levels of extracts from normal animals. This conclusion is at variance with that of Gibson and co-workers and our own findings. The difference appears to be attributable to the two different experimental approaches to this problem. Gibson's approach is through the more direct assay of the level of the enzymes involved in fatty acid synthesis, whereas the approach of Numa *et al.* is an indirect one, depending primarily on the ability of added acetyl CoA carboxylase to restore normal synthetic levels to a given amount of extracted protein.

There may also be another factor involved in the regulation of fatty acid synthesis and more precisely in the level of malonyl CoA. Malonyl CoA decarboxylase, an enzyme found in liver extracts which will decarboxylate malonyl CoA to acetyl CoA and CO₂ (206), may function as a regulatory agent by decarboxylating malonyl CoA, thus preventing it from being converted to fatty acids. It is not known whether the level of this enzyme is affected by starvation or diabetes and further information is necessary in order to ascertain the physiological role of this enzyme in fatty acid synthesis.

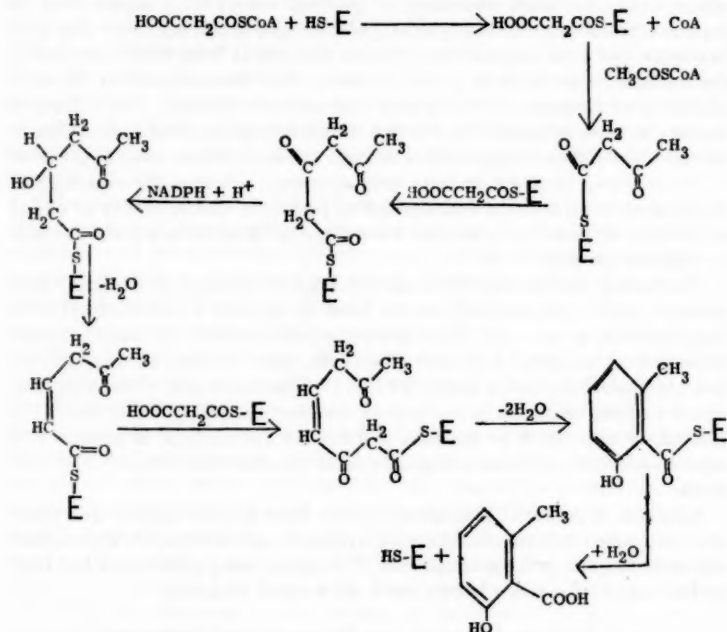
Catravas & Anker (32) isolated a factor from normal animals and yeast which increased the rate of acetate incorporation into fatty acids when added to liver homogenates from fasted rats. This factor was purified over 400-fold, but its exact nature is not known nor is the locus of its action.

SYNTHESIS OF AROMATIC AND POLYACETATE COMPOUNDS³

The biosynthesis of the aromatic nucleus of naturally occurring compounds can be achieved via two distinct pathways. The first is the shikimic acid pathway and involves the direct cyclization of carbohydrate derivatives (43). This system is shown to be the major pathway for the synthesis of phenylalanine and tyrosine in microorganisms (43).

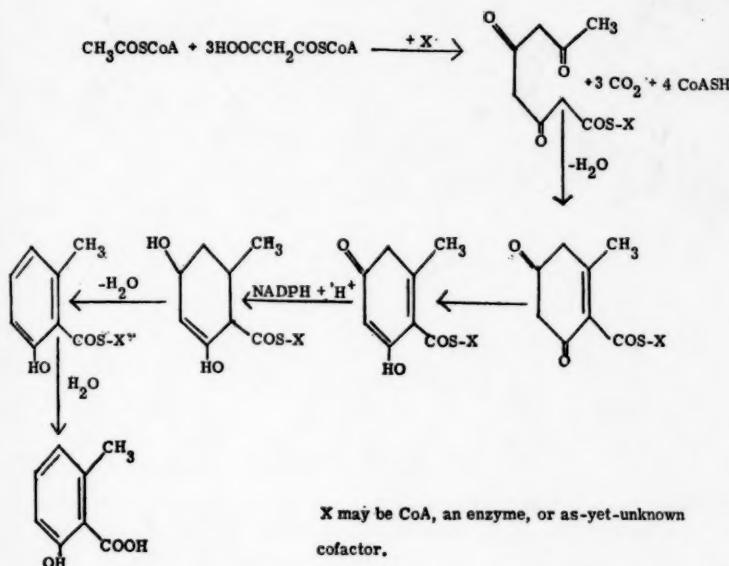
The second pathway for the synthesis of aromatic compounds appears to be the direct head-to-tail polycondensation of acetic acid first suggested by Collie (39) over fifty years ago. Recently, Birch (15) has developed this concept further and applied it to explain the biosynthesis of many natural products. Birch *et al.* (14) isolated 6-methylsalicylic acid from *P. griseofulvum* grown in the presence of C¹⁴-acetate. The methylsalicylic acid on degradation, was shown to derive all its carbon atoms from acetic acid by head-to-tail condensation. Similar results were obtained later by Tanenbaum & Bassett (8, 187) in their studies on the incorporation of C¹⁴-labeled acetate into 6-methylsalicylate by *P. patulum*. These authors were also able to prepare cell free extracts from the same mold that could convert C¹⁴-acetyl CoA to

³ For detailed background on the biosynthesis of aromatic compounds see Birch (15).



SCHEME IV. The Synthesis of 6-Methylsalicylic Acid

6-methylsalicylic acid (8). Bu'Lock & Smalley (30) were able to show that C^{14} -malonate can be converted to 6-methylsalicylic acid by *P. patulum*. Since malonyl CoA is the source of the multiple C_2 units for fatty acid synthesis (202, 206), it appeared most probable that malonyl CoA is also the condensing partner in the synthesis of the aromatic compounds. More recently, Lynen & Tada (120) reported on the conversion of C^{14} -acetyl CoA to 6-methylsalicylic acid in the presence of malonyl CoA, NADPH_2 , and soluble extracts from *P. patulum* prepared by Tanenbaum & Bassett's procedure (187). They found that cysteine and glutathione stimulated the incorporation into the 6-methylsalicylic acid, whereas iodoacetamide inhibited the synthesis. These observations indicated that the enzyme involved is a sulfhydryl enzyme similar to that involved in fatty acid synthesis. Using the same multi-enzyme complex concept that they have proposed for fatty acid synthesis, Lynen & Tada (120) presented the preceding hypothetical scheme (Scheme IV) for the synthesis of 6-methylsalicylic acid from malonyl CoA and acetyl CoA, dependent on the initial formation of malonyl-S-enzyme as the condensing partner.

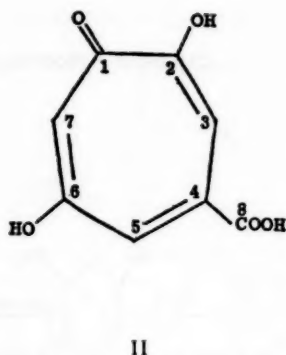
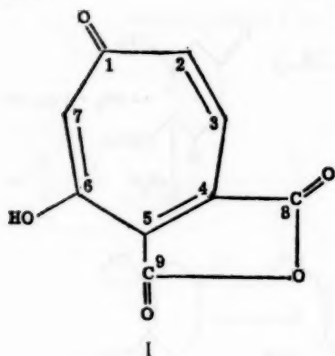


SCHEME V. Alternative Scheme for the Synthesis of 6-Methylsalicylic Acid.

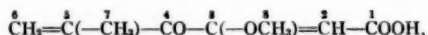
This scheme assumes that there is a multi-enzyme complex that carries on seven or eight different chemical reactions, that NADPH_2 is involved prior to cyclization, and that a *cis* α - β -unsaturated fatty acyl intermediate is formed. No information is available as to molecular weight of the enzyme complex nor is there any direct experimental evidence to support the notion of a fixed acyl-S-enzyme that can be made available to several different loci.

An alternative scheme can be proposed for the synthesis of 6-methylsalicylic acid which assumes the formation of a polyketo acyl derivative followed by condensation to form the six-membered ring, subsequent reduction, and dehydration. This scheme (Scheme V) is similar to that proposed for the formation of shikimic acid from 5-dehydroshikimic acid which is also a NADPH_2 -catalyzed reaction (43).

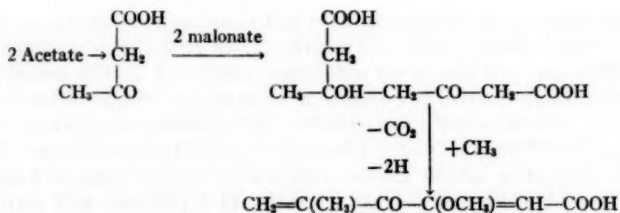
The conversion of malonate and acetate to tropolones were reported by Bentley (10, 11), who was able to obtain labeled stipitatic (I) and stipitatic (II) acids from *Penicillium stipitatum* cultures grown in the presence of C^{14} -acetate or C^{14} -malonate. Carbon atoms 2, 4, 6, and 9 are derived from the carboxyl carbon of acetate or malonate, whereas carbon atoms number 1, 3, 5, and 8 are derived from the methylcarbon of acetate or carbon number



2 of malonate. Carbon atom number 7 is derived from formate.
Labeled penicillic acid,



has been obtained by Keil (94) from *Penicillium cyclopium* cultures grown on C^{14} -acetate and C^{14} -malonate. Carbon atoms 2, 4, 6, and 7 are derived from methyl carbons of acetic acid, whereas carbon atoms 1 and 3 are derived from carbonyl carbons of malonic acid. It appears that two molecules of malonate are condensed with acetoacetate to form penicillic acid (Scheme VI).



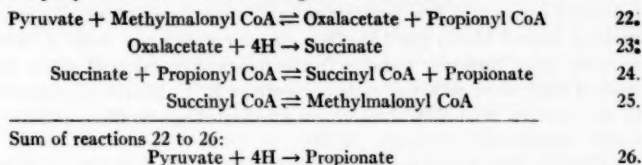
SCHEME VI. The Formation of Penicillic Acid.

Kaneda & Corcoran (89) reported on the incorporation of labeled methylmalonate into the erythronolide by washed cells of *Saccharomyces erythreus*, thus extending the reactions of malonate and its substituted derivatives into the synthesis of mycin from propionic acid. According to this mechanism, propionic acid is activated by thiokinase to propionyl CoA, which is then carboxylated to methylmalonyl CoA. The latter compound is condensed with propionyl CoA to form the methylated polyketo acid, which is then reduced

to form erthronolide. Lynen & Tada (120) also postulated a multi-enzyme complex for the aforementioned reactions.

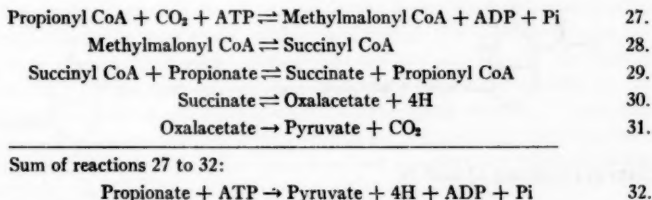
PROPIONIC ACID METABOLISM

The recent discovery by Swick & Wood (185) of a novel biochemical reaction, in which CO_2 is transferred from a donor compound to an acceptor without the expenditure of energy, provided the missing link in the metabolism of propionate by the propioni-bacteria. In these bacteria, pyruvate is converted to propionate via the following set of reactions:



The first and fourth reactions are catalyzed, respectively, by methylmalonyl oxalacetic transcarboxylase and methylmalonyl isomerase, whereas the others are catalyzed by well-known enzymes. Wood & Stjernholm (216) have isolated the transcarboxylase enzyme from *Propionibacterium shermanii* and purified it extensively. The enzyme has a broad specificity for the acyl CoA derivatives, whereas pyruvate is the only keto acid found as a carboxyl acceptor from methylmalonyl CoA. With oxalacetate as CO_2 donor, acetyl CoA, propionyl CoA, butyryl CoA, or acetoacetyl CoA may serve as acceptor. When acetyl CoA was used as an acceptor, malonyl CoA was formed, which suggested a possible role for this enzyme in fatty acid synthesis. The transcarboxylase was found to be a biotin-enzyme and could be readily inhibited by avidin.

The utilization of propionate by animal tissue follows essentially a similar sequence of reactions, except that the fixation of CO_2 requires the expenditure of energy in the form of ATP, as shown in the following reactions:

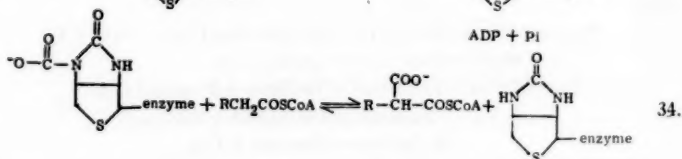


Reaction 27 is catalyzed by propionyl carboxylase and has been studied extensively by Ochoa and his co-workers (9, 53, 90, 91, 190) and by Lane *et al.* (72, 73, 74, 102, 105). Halenz & Lane (73) isolated propionyl carboxylase from ox liver mitochondria and studied its substrate specificity. The rate of carboxylation of propionyl, butyryl, and acetyl CoA were found to be 100,

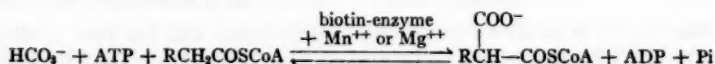
4.7, and 1.02, respectively. Kaziro *et al.* (91) crystallized the enzyme from pig heart and determined the molecular weight to be 700,000. Similar to the liver carboxylase, the crystalline pig heart enzyme can carboxylate propionyl CoA, butyryl CoA, crotonyl CoA, and acetyl CoA with relative rates of 100, 6, 3, and 1, respectively. Carboxylating enzymes with similar substrate specificity were found by Stern *et al.* (177) in dog skeletal and heart muscle and in ox, chicken, and rat liver.

Similar to acetyl carboxylase (207, 210), biotin comprises the prosthetic group of propionyl carboxylase and its enzymatic activity is completely inhibited by avidin. The crystalline propionyl carboxylase (91) contains one mole of bound biotin per 175,000 g, or four moles per mole of protein. The enzyme has a turnover number in the forward reaction of about 20,000 per mole of enzyme or 5000 per mole of biotin at 30°C. Biotin is covalently bound to the protein, probably through an amide linkage to the ϵ -amino groups of lysine residues as in biocytin (91, 207).

Enzyme-biotin- CO_2 complex is now accepted as the form of the intermediate resulting during biotin-catalyzed carboxylation reactions (91, 105). The earlier proposed mechanisms (74, 117) for the events leading to the formation of the enzyme- CO_2 that involved the intermediate formation of enzyme-ADP or enzyme- PO_4 have been shown to be untenable. Available evidence supports the direct concerted formation of enzyme- CO_2 from ATP and CO_2 (91, 105). The enzyme- CO_2 complex can be regarded as an activated carbonic acid, probably bound onto the enzyme biotin as the N-carboxy-biotin-enzyme derivative (120). The activated CO_2 is then transferred to an α -carbon of an acyl acceptor to form the corresponding derivative of malonyl CoA as shown in the following sequence of reactions:



Sum of reactions 33 and 34:



R can be H as in acetyl CoA, methyl groups as in propionyl CoA, or ethyl group as in butyryl CoA.

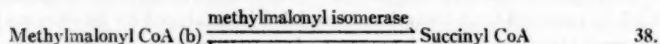
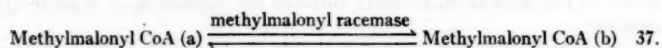
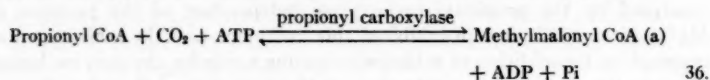
Halenz & Lane (73) and, independently, Friedman & Stern (55) presented direct evidence in support of reaction 34. Both workers showed that the exchange between 1-C¹⁴-propionyl CoA and methylmalonyl CoA could be catalyzed by the propionyl carboxylase independent of the presence of Mg⁺⁺, Pi or ADP. Avidin inhibited this exchange, and the inhibition was reversed by the addition of D-biotin indicating a role for the enzyme-bound biotin in this exchange, possibly through the formation of N-carboxybiotin-enzyme.

Methylmalonyl CoA isomerase.—The isomerization of methylmalonyl CoA to succinyl CoA (reaction 25 and 28) is catalyzed by an enzyme known as methylmalonyl CoA isomerase. Interest in this enzyme was greatly stimulated when Smith & Monty (165) reported that the isomerase activity in B₁₂-deficient rat livers was much lower than in livers from normal rats. Soon thereafter, Garnani *et al.* (58) were able to demonstrate that the B₁₂-coenzyme (dimethylbenzimidazolyl cobamide) could restore the isomerase activity of liver extracts of deficient animals. The stimulating effect of the B₁₂-coenzyme on the isolated enzyme was demonstrated by Stern & Friedman (176) on an ammonium sulfate fraction from ox liver extracts, and by Stadtman *et al.* (171) on charcoal-treated extracts of propionibacteria. Lengyel *et al.* (112) were able to resolve the isomerase from kidney enzyme by acidification in the presence of ammonium sulfate and to reactivate the apoenzyme by dimethylbenzimidazolylcobamide ($K_m = 2.1 \times 10^{-8} M$) and by benzimidazolylcobamide ($K_m = 2.0 \times 10^{-7} M$), but not by adenylobamide. Stjernholm & Wood (181) purified the isomerase from *Propionibacterium shermanii* and found an absolute requirement for the cobamide coenzyme. The equilibrium constant for the methylmalonyl CoA isomerase reaction was studied and the ratio of (succinyl CoA)/(methylmalonyl CoA) was found to be 10.5 at pH 7.0 and 25°C.

Eggerer *et al.* (52) investigated the mechanism of the cobamide coenzyme in the isomerization of methylmalonyl CoA using 2-C¹⁴-methylmalonyl CoA and an isomerase preparation from *P. shermanii*. They found that 80 per cent of the succinyl CoA formed was labeled at the carbon 3 position, indicating that an intermolecular transfer of the thiolester group had occurred during the isomerization. On this basis, and in analogy to the intramolecular rearrangement of phenylneopentyl radical, the authors proposed a free radical formation from methylmalonyl CoA produced by a one-electron oxidation of the substrate by Co⁺³ of the coenzyme. The validity of this mechanism can be tested by the incorporation of a proton from the medium into succinyl CoA, since according to this hypothesis a proton is reversibly released to the medium during the isomerization.

Recently, Mazumder *et al.* (130) were able to split the methylmalonyl CoA isomerase activity of sheep liver extracts into two separate protein fractions which are required for the isomerization of the enzymatically synthesized methylmalonyl CoA to succinyl CoA. Available evidence suggests that one of the enzyme fractions is concerned with the racemization of the

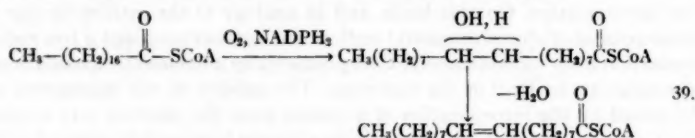
methylmalonyl CoA to the correct isomer while the other fraction is concerned with the isomerization of the correct isomer of the methylmalonyl CoA to succinyl CoA. The following sequence of reactions is suggested:



Methylmalonyl CoA (a) and (b) represent the two isomers of this compound

SYNTHESIS OF UNSATURATED FATTY ACIDS

The direct conversion of saturated fatty acids to unsaturated fatty acids was first demonstrated by Schoenheimer & Rittenberg (156, 179) in their classical work on the conversion of deuterated fatty acids by mice and rats. Though many workers (87, 88, 106 to 109, 159) have reported on the direct desaturation of fatty acids by crude enzyme preparations, using the methylene blue assay, no direct and adequate experiments were presented to reveal the exact mechanism of such transformation. Recently, Bloomfield & Bloch (17, 18) reported the oxygen dependent desaturation of 1-C¹⁴-palmitic and 1-C¹⁴-stearic acids to palmitoleic and oleic acids, respectively, by both whole yeast cells and their extracts. Palmityl CoA was shown to be the substrate which, in the presence of a particulate fraction of yeast homogenates, NADPH₂, and oxygen, could be desaturated to palmitoleyl CoA. These requirements were characteristics of oxygenase reactions and prompted the authors to postulate oxy acids as intermediates. This was later substantiated by Lennarz & Bloch (113) who were able to show that 9-hydroxystearic acid was converted to an olefinic acid by yeast extract and that both 9- and 10-hydroxystearic acids can promote the growth of yeast anaerobically. On the basis of these observations, Lennarz & Bloch proposed the following sequence of reactions for the desaturation of fatty acids by yeast.

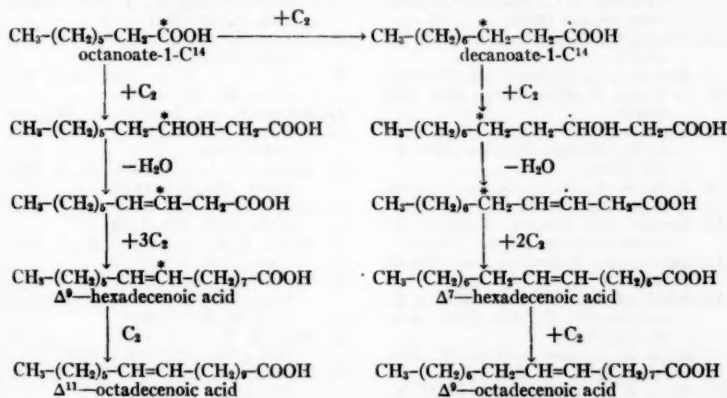


Recently, Bernhard *et al.* (12) studied the *in vitro* conversion of radio-labeled stearate to oleate in animal tissue and found that both ATP and oxygen were required for this conversion, thus substantiating Bloomfield & Bloch's (18) observations in the yeast system. Though stearic acid under similar but anaerobic conditions could reduce methylene blue, no oleic acid was formed. Mudd & Stumpf (136) reported on the biosynthesis of oleate

from acetate by particulate preparations from avocado mesocarps. Oxygen was absolutely required for oleate synthesis. Oleic acid synthesis in avocado particles appears to be a *de novo* synthesis from acetate (136), and attempts to demonstrate direct desaturation of stearate in this system were unsuccessful because of the immediate esterification of the added acid into triglycerides and phospholipids.

The biosynthesis of linoleic acid appears to be accomplished by further desaturation of oleic acid. Yuan & Bloch (217) were able to demonstrate the direct transformation of C^{14} -oleic acid to linoleic and linolenic acids by cells of *Torulopsis utilis*. This transformation requires oxygen and appears to be a direct desaturation of oleic acid. Therefore, the introduction of the double bond into carbon atoms 12 and 13 of oleic acid is oxidative in nature and is analogous to the oxidative desaturation of stearate to oleate.

Synthesis of unsaturated fatty acids by anaerobic organisms.—Since oxygen has been found to be an indispensable component in the formation of Δ^9 unsaturated fatty acid, it became of interest to find out how microorganisms that are strictly anaerobic synthesize unsaturated acids. Goldfine & Bloch (69) studied the synthesis of unsaturated fatty acids in *Clostridium butyricum* and *Cl. kluyveri* and found that C^{14} -labeled palmitic and stearic acids were not converted to the unsaturated analogues, which indicated that long-chain fatty acids are not the direct precursors of olefinic acids in these organisms. Shorter-chain fatty acids (C_8 , C_{10} , C_{12} , C_{14}) underwent chain elongation, but only octanoic-1- C^{14} and decanoic-1- C^{14} incorporated on elongation into both saturated and unsaturated fatty acids. No randomization of the radioactivity takes place during the conversion of these acids to the unsaturated fatty acids. Similar observations were reported by Reiser & Murty (148) on the biosynthesis of octadeca-9, 12-dienoic acid (linoleic acid) from *cis*-2-octanoic acid by the laying hen. More recently, Scheuerbrandt *et al.* (155) found that



SCHEME VII. Anaerobic Synthesis of Unsaturated Fatty Acids.

when *Cl. butyricum* was grown in a medium containing octanoate-1- C^{14} , the unsaturated fatty acids (hexadecenoic and octadecenoic acids) contained the double bonds between carbon atoms 7 and 8 (counting from the methyl end of the molecule), whereas in the presence of decanoate-1- C^{14} as precursor the unsaturated fatty acids contained the double bonds between carbon atoms 9 and 10 (counting also from the methyl end of the molecule). These observations indicated that the double bonds are introduced early during the synthesis of unsaturated fatty acids and that there is no direct interconversion of double bond isomers. Similar conclusions were reached earlier by Hofmann and co-workers (76) from their studies on growth requirements for lactobacilli in biotin-free media. On the basis of the foregoing observations, Scheuerbrandt *et al.* (155) proposed the following mechanism for the anaerobic synthesis of unsaturated fatty acids (Scheme VII).

ACKNOWLEDGMENTS

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CARBOHYDRATE METABOLISM^{1,2}

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Space limitations require that certain aspects be emphasized in these reviews. Although in the present article an attempt has been made to include most of the important recent developments in the assigned area, particular attention has been given to metabolic pathways in mammals. Relevant or especially significant work on plants and microorganisms has often been included, but for more complete coverage the reader is referred to the *Annual Review of Plant Physiology* and *The Annual Review of Microbiology*.

GLYCOGEN AND STARCH

Glycogen.—Studies of glycogen metabolism are generally based on the now-accepted concept that the glycogen-synthesizing enzyme is UDPG-glycogen transglucosylase (glycogen synthetase) and that phosphorylase is the enzyme responsible for glycogen breakdown. Basu & Bachhawat (1) have purified the synthetase from sheep brain and found that its activation by glucose-6-phosphate is comparable to the activation of the muscle enzyme but markedly lower than that of the liver enzyme. Furthermore, glucose-6-phosphate was the only sugar phosphate showing this activating property. Breckenridge & Crawford (2) also purified the rabbit brain enzyme and concluded that there is sufficient enzyme to account for glycogen synthesis in brain *in vivo*. The enzyme required for the formation of UDPG, namely, UDPG pyrophosphorylase, has also been purified from brain (3) and a method devised for its estimation in micro amounts (4). The distribution of glycogen synthetase, UDPG pyrophosphorylase, phosphorylase, and phosphoglucomutase in eight different regions of the rabbit central nervous system has been determined (5).

Nigam & MacDonald (6) attribute the low glycogen storage in tumors to a low level of the transglucosylase accompanying normal levels of phosphorylase. This is in disagreement with the work of Nirenberg (7, 8), which has been confirmed by Inouye & Oikawa (9). The latter authors found depressed levels of phosphorylase in tumors as well as in tissues having a high rate of cell division. However, the same tumors were not used in the conflicting

¹ The survey of the literature pertaining to this review was concluded in September 1961.

² The following abbreviations are used: NAD for nicotinamide adenine dinucleotide; NADP for nicotinamide adenine dinucleotide phosphate; NADPH₂ for nicotinamide adenine dinucleotide phosphate, reduced form; UDP for uridine diphosphate; UDPG for uridine diphosphate glucose; UDPGA for uridine diphosphate glucuronic acid.

investigations, although hepatomas of various types were included in the studies.

In confirmation of the implications of earlier work, a detailed electron microscopic and biochemical study by Luck (10) on glycogen obtained by fractional centrifugation of liver homogenates demonstrated that UDPG-glycogen transglucosylase is bound to the polysaccharide, rather than to microsomal membranes that accompany the glycogen when it is isolated by the usual methods of fractional centrifugation. A histochemical investigation of individual fibers of resting rat skeletal muscle indicated that the phosphorylase and the transglucosylase enzyme systems have a reciprocal relationship: that is, the former is predominant in large fibers and the latter in small fibers (11). These observations were considered to be inconsistent with the concept that the two enzymes act as parts of a single enzymatic cycle.

Uridine diphosphate glucose-glycogen transglucosylase has also been isolated from microorganisms. The enzyme purified from yeast showed only a low order of activation by glucose-6-phosphate (12), while synthetase extracted from *Agrobacterium tumefaciens* was not stimulated at all (13).

There is naturally great interest in hormonal effects on glycogen synthetase. Belocopitow (14) reported that the enzyme is inhibited somewhat by epinephrine via a mechanism involving cyclic-3',5'-adenylic acid. Steiner *et al.* (15) found that synthetase activity in the liver of alloxan-diabetic rats is elevated, provided that added glucose-6-phosphate is used in the assay mixture, and that treatment of the diabetic animals with insulin causes a marked increase in synthetase activity within two hours after injection. Prednisolone increases hepatic glycogen and glucose-6-phosphate, but not synthetase activity, while glucagon has little effect on any of these. The findings emphasize that glycogen synthesis may be controlled by the concentration of the synthetase or of its activator, glucose-6-phosphate, as originally suggested by Leloir & Goldemberg (16), and recall the insulin-induced activation of glycogen synthetase in rat diaphragm observed by Villar-Palasi & Lerner (17).

The crystallization of phosphorylases *a* and *b* from human autopsy skeletal muscle has been achieved by Yunis *et al.* (18). The preparations appeared to be identical, even immunologically, with the enzymes derived from rabbit muscle.

Brown, Illingworth & Cori (19, 20) have reported their detailed investigation of the *de novo* synthesis of polysaccharide by phosphorylase, a process which might be important during embryological development. Using very highly purified enzyme and substrate, they found that amylose is slowly formed from glucose-1-phosphate in the absence of primer. In the complete absence of branching enzyme, there is an absolute lag period during which there is no phosphate liberation or demonstrable exchange of inorganic P^{32} . A more rapid reaction that follows is recognizable by liberation of inorganic phosphate and reaction with iodine. The product formed has more than 2000 glucose residues.

Anoxia has been shown by Cornblath *et al.* (21) to induce in the perfused rat heart a very rapid glycogenolysis associated with a partial conversion of phosphorylase *b* to *a*. In connection with a study of the mechanism by which various hormones stimulate the release of free fatty acids from adipose tissue, Vaughn (22) has shown that ACTH, epinephrine, norepinephrine, glucagon, and serotonin increase the phosphorylase activity of this tissue *in vitro*. However, an unexpected finding of considerable potential importance made by Lyon (23) is that glycogen of the gastrocnemius and abdominal muscles of the I strain of mice (but not the C57 strain) can apparently yield glucose in the absence of the active form of phosphorylase. Preincubation with ATP, cyclic-3',5'-adenylic acid, pyridoxal phosphate, or pyridoxamine phosphate, alone or in combination, did not effect the formation of the active form. Furthermore, injections of epinephrine induced glycogenolysis as well as increases in blood glucose and lactate, but the active enzyme could still not be detected. These extremely interesting results require further study for a fuller understanding of glycogenolysis *in vivo*.

On the basis of the observation that isolated rat liver cells produce glucose by a mechanism apparently indifferent to various added cofactors, including hormones, Rutter and co-workers (24, 25, 26) investigated the breakdown of glycogen by the combined action of liver amylase and serum oligosaccharase (maltase). The liver amylase is primarily associated with microsomal particles and was characterized as an α -amylase with a specificity similar to those of other mammalian enzymes of this type (24). From the fact that various treatments of the microsomes led to marked increases in activity, it was concluded that the liver enzyme is integrated within the particles in a configuration such that the active sites are screened or that a diffusion barrier is presented to large substrate molecules. The insensitivity of rat liver slices and isolated cells to epinephrine and glucagon, as well as other abnormalities of such preparations, led Rutter *et al.* to be appropriately cautious in estimating the importance of the amylase pathway from studies on these preparations. Further study of the problem with the isolated, perfused liver of the rat, a species which has a much higher amylase level in liver than other mammals, finally led to the conclusion that the liver amylase-serum oligosaccharase system is not quantitatively significant for the production of serum glucose (26). However, the studies lent support to the idea that the normal rat liver is a source of serum amylase.

Olavarria (27, 28) has carried out a study, closely related to Rutter's, on the origin of the oligosaccharides of liver. Through the use of UDP-glucose- C^{14} he has shown that these substances are labeled more slowly than glycogen, the data indicating that they are derived from degradation of newly formed exterior chains of glycogen. This conclusion does not support the suggestion (29) that the oligosaccharides may be precursors of the precursors of the polysaccharide. Torres & Olavarria (28) identified two glycogen-degrading enzymes in muscle and liver extracts, an α -amylase (particle-bound in liver and perhaps in muscle), and a glucose-forming enzyme.

Fasting of rats leads to a decrease in liver phosphorylase, the original level being restored by refeeding for approximately the same period of time as the previous fasting period (30). Large amounts of liver glycogen have been found at the end of gestation in fetuses of all species that have been examined (31). The glycogen disappears rapidly after birth. In explanation of this phenomenon, Dawkins (32) has found, in four species (rat, rabbit, guinea pig, and sheep) differing greatly in the maturity of their new born, that the hepatic glucose-6-phosphatase levels are much higher 24 hours after birth than in the fetus at birth or in the adult. This held regardless of the mode of delivery or duration of pregnancy (i.e., Caesarian section two days before term or prolongation of pregnancy by the administration of chorionic gonadotropin). However, in the kidney of the rat, the extremely rapid disappearance of glycogen immediately after birth is not accompanied by an appreciable change in activity of glucose-6-phosphatase (33).

Roe *et al.* (34) have reported that there are not two types of liver glycogen differing in extractability and that, with adequate homogenization, all of the glycogen in tissues can be extracted with trichloroacetic acid solution. Lowe & Garner (35) have presented evidence that liver glycogen isolated by mild techniques contains RNA fragments. Purines, but not pyrimidines, could be liberated from this complex.

Starch biosynthesis.—Although the biochemistry of starch cannot be covered in this review, one outstanding development must be mentioned. The association of glycogen synthetase with glycogen particles, as well as many other considerations concerning polysaccharide synthesis in plants and animals, has led Leloir and his colleagues (36, 37) to examine starch granules for a starch-synthesizing system based on UDPG. They have succeeded in finding such a system in the starch granules of young potatoes, sweet corn, and immature dwarf string beans. The bean granules were shown to catalyze the transfer of labeled glucose from UDPG to the starch fraction, as indicated by the disappearance of UDPG, formation of UDP, incorporation of labeled glucose into the polysaccharide, and degradation of the labeled starch with α -amylase. The starch-synthesizing enzyme, which has not successfully been extracted from the granules, apparently catalyzes the addition of glucose to di- and oligosaccharides of the maltose series as well as to starch. Both amylose and amylopectin were found to contain the labeled glucose, but it was not possible to determine whether both polysaccharides participate in the transfer reaction, or whether one is the precursor of the other. The very high K_m value (6×10^{-2}) for UDPG may reflect the existence of diffusion barriers, since the enzymatic system could not be studied in soluble form.

For a detailed discussion of enzymological aspects of the metabolism of both starch and glycogen, the paper by Whelan (38) may be consulted.

MUCOPOLYSACCHARIDES AND SOME OF THEIR CONSTITUENTS

During the past year there have been many interesting developments in this area, particularly in biosynthetic mechanisms. The mechanism of forma-

tion of sialic acids and 6-deoxyhexoses now appears to have been established, and much has been learned about the incorporation of sulfate. Work on these three polysaccharide constituents will therefore be discussed in some detail.

Sialic acids.—It should first be mentioned that Gottschalk's book on the chemistry and biology of the sialic acids and related compounds has appeared (39).

The understanding of the biosynthesis of the sialic acids must rest on reliable knowledge of the formation of hexosamine precursors. Detailed studies have now been published on purified L-glutamine-D-fructose-6-phosphate transamidase obtained from *Escherichia coli*, *Neurospora crassa*, and rat liver (40). The preparations show specific requirements for L-glutamine and D-fructose-6-phosphate for the synthesis of glucosamine-6-phosphate. The relationship of this system to the one employing ammonia in

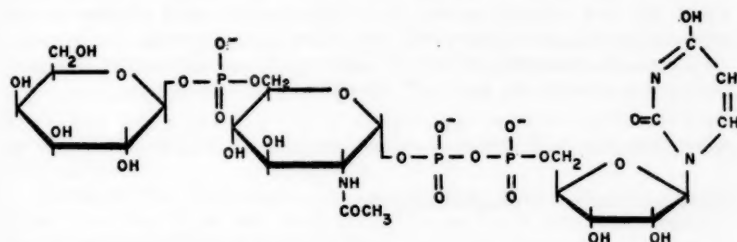


FIG. 1.—Probable formula of isolated nucleotide sugar containing sugars linked through phosphodiester group.

place of glutamine is discussed in this paper. Gryder & Pogell (41) have confirmed their own findings that glucose-6-phosphate is more active than fructose-6-phosphate for the synthesis of glucosamine-6-phosphate in the rat liver system using L-glutamine. They discussed possible explanations for the apparent conflict between their results and those of Roseman's group (40) in regard to the nature of the hexose-6-phosphate substrate. Various aspects of the biosynthesis of amino sugars by intestinal mucosa are being investigated (42).

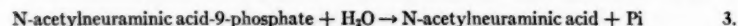
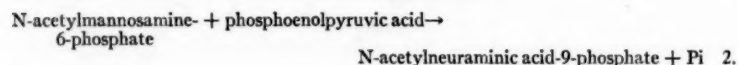
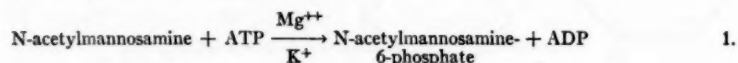
The acetylation of glucosamine-6-phosphate and its conversion to uridine nucleotide derivatives are well known. However, it is relevant that evidence has recently been obtained for the presence of N-formylhexosamine in an abnormal urinary glycoprotein (43). A new glucosamine-containing nucleotide, shown in Figure 1, was discovered by Suzuki (44) in the oviduct of the laying hen. The unique phosphodiester group linking two sugars has not previously been encountered in natural products.

Since sialic acids (acyl-substituted neuraminic acids) are mannosamine derivatives, mechanisms must be available for the epimerization of glucos-

amine derivatives. Roseman and his co-workers have already reported that rat liver extracts catalyze the conversion of UDP-N-acetylglucosamine to UDP and N-acetylmannosamine (45) and that an epimerase from *Acetobacter cloacae* effects the interconversion of N-acetyl-D-glucosamine-6-phosphate and N-acetyl-D-mannosamine-6-phosphate (46). Further work is necessary in this area, particularly since enzymatic studies on hexosamine metabolism frequently give evidence for the existence of alternative pathways.

In regard to the formation of the sialic acids, a major breakthrough has been made by Warren & Felsenfeld (47) in their discovery of an enzyme system in liver that catalyzes the synthesis of N-acetylneuraminic acid from N-acetylmannosamine, phosphoenolpyruvic acid, and ATP. The key to their success was the use of mild homogenization techniques in the preparation of the liver extracts. Previously, Comb & Roseman (48) had reported the synthesis of N-acetylneuraminic acid from N-acetylmannosamine and pyruvic acid by an enzyme-catalyzed aldol condensation, but, unlike the new enzyme system, N-acetylneuraminic acid aldolase favors degradation of N-acetylneuraminic acid rather than synthesis.

Warren & Felsenfeld (49) and Roseman and his co-workers (50, 51) have been able to separate the new synthetic pathway into three steps:



The kinase has been purified from rat liver (49, 50) but is also present in bovine submaxillary gland (49). The synthetase producing N-acetylneuraminic acid-9-phosphate has been purified from submaxillary glands (49, 51). N-Acetylmannosamine and N-acetylmannosamine-6-phosphate can be replaced by their corresponding N-glycolyl derivatives, but it is still premature to assume that one kinase and one synthetase can act on both the N-acetyl and N-glycolyl derivatives. The kinase system is specific for the mannosamine derivatives (50).

The mechanism for the incorporation of sialic acid into mucopolysaccharides has not been elucidated. Exogenous sialic acid is not utilized and is excreted in the urine (52). Cytidine monophosphate-N-acetylneuraminic acid has been found in *E. coli* K-235 (53); and, more recently, preliminary evidence has been presented by Jourdain *et al.* (54) for the occurrence in goat colostrum of uridine diphosphate derivatives of trisaccharides containing galactose, N-acetylglucosamine, and N-acetylneuraminic acid. The occurrence of the nucleotide trisaccharides suggests that carbohydrate residues might be added as oligosaccharide groups, rather than as individual mono-

saccharides, in the formation of complex glycoproteins, glycolipids, and heteropolysaccharides.

Blix & Lindberg (55) have carefully studied the sialic acids in hydrolysates of bovine and equine submaxillary mucins. The bovine mucin yielded five sialic acids, the predominant ones being N-acetyl-7-O-acetylneuraminic acid and N-acetyl-di-O-acetylneuraminic acid (O-acetyl on C-7 and on C-8 or C-9). Also isolated were N-glycolylneuraminic acid, N-acetylneuraminic acid (perhaps derived from the diacetyl compound), and a fifth derivative suggested to be a labile isomer of the diacetylneuraminic acid. The equine submaxillary mucin yielded N-acetyl-4-O-acetylneuraminic acid. However, a report (56) that serum gonadotropin contains three different sialic acids (N-acetylneuraminic acid, N-glycolylneuraminic acid, and an unidentified one) could not be confirmed (57). Only the N-acetyl derivative was found.

With few exceptions, sialic acids are found in oligosaccharides and in complex high molecular substances ("sialopolymers"). A notable exception encountered by Warren (58) is the very high concentration of free sialic acids (both N-glycolyl- and N-acetylneuraminic acids) in trout eggs, the free sialic acid content far exceeding that of free N-acetylhexosamine. In chick egg white, however, a detailed examination revealed that all the sialic acid is bound to proteins (59). Carubelli *et al.* (60) have isolated a new sialic acid containing oligosaccharide, neuramin-lactose sulfate, from the mammary gland of the rat.

Studies on the biosynthesis of sialopolymers require information regarding the location of the sialic acid residue. Experiments with neuraminidase are sometimes difficult to interpret in regard to the estimation of terminal sialic acid. Release of the sugar by the enzyme is almost always far from quantitative [50 to 60 per cent from chick egg yolk membranes (59), 70 per cent from haptoglobin (61)], but enzymatic hydrolysis of serum gonadotropin with neuraminidase liberated almost 100 per cent of the N-acetylneuraminic acid released by acid hydrolysis (57). When the sialic acid is bound by a linkage that is not susceptible to neuraminidase, it is of interest to know whether or not the acid occupies a terminal position. Chargaff and co-workers (62, 63), on the basis of nonenzymatic methods of degradation, have reported that only half the sialic acid in ox brain glycolipid is terminal.

The mode of attachment of the terminal sialic acid has had some study. A sialomucoprotein has been liberated by trypsin from the human erythrocyte (64; see also 65, 66). The α -D-N-acetylneuraminyl (2 \rightarrow 6) N-acetyl-galactosaminyl group of ovine submaxillary mucoprotein has been reported by Gottschalk (67, 68) to be attached mainly by glycosidic-ester linkages to the free carboxyl groups of aspartyl and glutamyl residues and perhaps partly by O-glycosidic linkages to hydroxy amino acid residues. Similar linkages apparently occur in bovine submaxillary mucoprotein as well (69). As yet, only N-acetylglucosamine and galactose have been shown to be partners of the terminal sialic acid in sialopolymers, the linkage involving the C-3

hydroxyl of galactose, as seems likely in orosomucoid (70), and the C-6 hydroxyl of N-acetylglucosamine, as in submaxillary gland mucoprotein (71, 72).

Progress in elucidating physiological functions of sialic acids will be aided by information regarding their intracellular distribution and factors affecting this distribution. Wolfe (73), in a study of the distribution of gangliosides in subcellular fractions of guinea pig cerebral cortex, estimated the isolated gangliosides using a sialic acid assay procedure. The glycolipids were in highest concentration in microsomes and in other particles which are apparently rich in cell membranes. Patterson & Touster (74) found sialic acid in all fractions of rat liver except the nuclei, with mitochondrial and microsomal membranes being especially rich in the sugar. It is well known that sialic acid is commonly found on cell surfaces, such as the red blood cell membrane, and Sjöstrand (75) has suggested that mitochondrial membranes may consist in part of glycoprotein. It is not unlikely that such a substance would contain sialic acid. Recent studies of interest include those on the relationship of sialic acids to the electrokinetic charge on the human erythrocyte (76); on the correlation of structure, shape, and function of a salivary mucopolysaccharide (67); and on the ability of sialopolymers to restore the electrical excitability of cerebral tissues (77). The ability of sialopolymers to stimulate the growth of HeLa cells (but not fibroblast-B cells) in tissue culture is lost when the polymers are pretreated with neuraminidase (78). The relation of sialic acids to Rh (D) antigen has been studied (79). Although tumor-bearing animals may have elevated levels of sialic acid-containing glycoproteins, analysis of normal and tumor cells disclosed no significant difference in their content of hexosamine or sialic acid (80).

Methylpentoses (6-deoxyhexoses).—An important recent achievement has been the elucidation of biosynthetic pathways for both L-fucose and L-rhamnose. It has been demonstrated that certain bacteria convert labeled D-glucose to L-fucose without cleavage or inversion of the carbon chain (81, 82, 83), and this is true for man as well (84). The isolation of guanosine diphosphate L-fucose from ewe's milk (85) and from *Aerobacter aerogenes* (86) provided the clue to the mechanism of the transformation. In 1958 Ginsburg (87, 88) found that extracts of *A. aerogenes* convert guanosine diphosphate-mannose to guanosine diphosphate-L-fucose in the presence of added NADPH₂, and more recently he obtained evidence that guanosine diphosphate-4-keto-6-deoxy-D-mannose is an intermediate in the transformation (89, 90). In the absence of NADPH₂, this substance accumulated and was converted by catalytic hydrogenation to identifiable 6-deoxyhexoses. By a double enolization, the intermediate is presumably transformed into guanosine diphosphate-4-keto-L-galactose, which probably is the substrate of the NADPH₂-linked reduction. The biosynthetic route is shown in Figure 2.

Guanosine diphosphate sugars, including mannose and perhaps fucose, have been isolated from the lactating mammary gland (91). Extracts also

contain guanosine diphosphate-glucose pyrophosphorylase activity. Guanosine diphosphate-mannose has recently been found in a marine red alga, *Porphyra perforata* (92). Two microbial substances with structures related to L-fucose, namely, 5-keto-6-deoxy-D-arabohexose of hygromycin A (93) and colitose (3-deoxy-L-fucose) (94), have also been shown in tracer experiments to be formed from D-glucose without cleavage or inversion of the carbon chain.

Levvy has continued his study of mucopolysaccharide-splitting enzymes with the demonstration that α -L-fucosidase activity is present in a wide variety of mammalian species and tissues (95). The enzyme was highest in

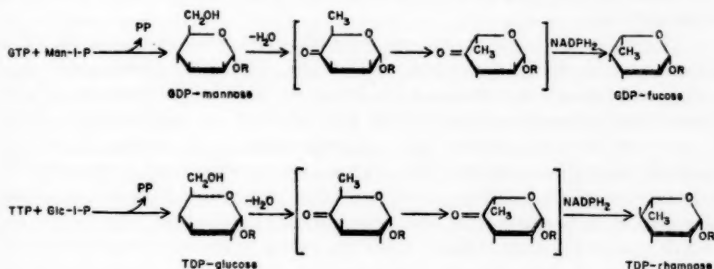


FIG. 2.—Biosynthetic pathways of guanosine diphosphate fucose and thymidine diphosphate rhamnose. NAD is required for the formation of keto intermediates but its mechanism of action has not been explained. Abbreviations are: GDP (guanosine diphosphate); GTP (guanosine triphosphate); $NADPH_2$ (nicotinamide-adenine dinucleotide phosphate (reduced form)); TTP (thymidine triphosphate); TDP (thymidine diphosphate).

rat epididymus, although the value was exceeded by that for the limpet, *Patella vulgata*. β -D-Fucosidase, but no β -L-fucosidase, activity was also detected in mammalian tissues. The amount of the former enzyme, as well as of several other glycosidases of cow ovary, was found to be proportional to the amount of luteal tissue present (96).

Kent & Mah (97) have reported briefly on the ATP-dependent utilization of L-fucose by extracts of sheep intestinal mucosal cells.

The trail of research in elucidation of the mechanism of formation of rhamnose was similar to that already described for fucose. Thymidine diphosphate-rhamnose was found in a variety of microorganisms (98 to 102), and it was shown that the conversion of glucose to rhamnose does not involve inversion of the carbon chain or randomization of a C^{14} label (103). Pazur & Shuey (104) found that cell-free extracts of *Streptococcus faecalis* convert thymidine triphosphate and glucose-1-phosphate into thymidine-diphosphate-glucose and then into thymidine-diphosphate-rhamnose. Simultane-

ously, Kornfeld & Glaser (105) discovered similar enzymatic systems in *Pseudomonas aeruginosa*, an organism that produces a rhamnose-containing glycolipid. Extensions of these studies (106, 107, 108), particularly the isolation and characterization of thymidine-diphosphate-4-keto-6-deoxyglucose by Glaser (109), have led to the formulation of the transformation shown in Figure 2. The sequence of reactions apparently also occurs in *E. coli* (110). Each step has been studied individually by the use of purified enzymes or by the omission of a coenzyme, experiments with tritiated water providing evidence for the enolizations involving C-3 and C-5 of the keto intermediate (109). NADPHH³, on the other hand, presumably labeled C-4, since all of the other carbon atoms of the labeled rhamnose produced were devoid of radioactivity.

Thymidine-diphosphate-glucose pyrophosphorylase has been found in rhamnose-containing organisms and plants, including germinated plant seeds, and was not detected in animal tissues (106). In *Streptomyces griseus*, thymidine-diphosphate-rhamnose is formed from thymidine-diphosphate-glucose and from thymidine-diphosphate-mannose, both of which have been isolated from this organism (102). The glucose and mannose nucleotides are interconverted by cell-free extracts (111). Thymidine-diphosphate-ribose has also been isolated from this organism (111). It should be noted that Smith *et al.* (112) have isolated UDP-rhamnose from nonencapsulated variants of type II pneumococcus, capsulated forms of which produce a rhamnose-containing polysaccharide.

The study of Taylor & Juni (113) on the biosynthesis of the capsular polysaccharide of a Gram-negative coccus (114, 115) is consistent with the hypothesis that the glucose chain is used without cleavage for the production of rhamnose. The organism possesses the Embden-Meyerhof and the Entner-Doudoroff pathways, but no hexokinase (114). Glucose-1-C¹⁴ and glucose-2-C¹⁴ were converted to unlabeled rhamnose, whereas glucose-6-C¹⁴ yielded polysaccharide glucose and rhamnose (labeled in C-1 and C-6) containing twice the specific activity of the substrate hexose. It therefore seems likely that a triose derived from C-4, C-5, and C-6 via the Entner-Doudoroff pathway is converted by Embden-Meyerhof transformations to hexose phosphate precursors of the monosaccharides in the polymer.

Sulfate.—The mechanism of this important process has been quite elusive, but it now appears to be in hand. The original observation of D'Abramo & Lipmann (116) on the incorporation of S³⁵ from S³⁵-sulfate and S³⁵-labeled 3'-phosphoadenosine-5'-phosphosulfate into chondroitin sulfate in an extract of embryonic chick cartilage has been followed by similar studies on mucopolysaccharides of hen oviduct (117 to 120), on heparin formation in extracts of mouse mastocytomas (121, 122), and on chondroitin sulfate formation in particle-free extracts of rat liver (123).

Suzuki & Strominger (117 to 120) found that "mucopolysaccharide sulfotransferase" of hen oviduct introduces sulfate into acetylgalactosamine and into oligosaccharides derived from chondroitin sulfate by enzymatic

hydrolysis (119), as well as into mucopolysaccharides (118, 119). Moreover, introduction of two sulfate residues into the same hexosamine residue led to the suggestion that the accepted structure of chondroitin sulfate may represent only a statistical model of a compound consisting of some unsubstituted and disubstituted galactosamine residues (120). The isthmus of the hen oviduct, which was used in these investigations, also contains uridine diphosphate N-acetylgalactosamine sulfate, but no function for this substance in the transfer of sulfate to mucopolysaccharides has been found (118). Exogenous N-acetyl-D-glucosamine 6-O-sulfate-S³⁵ is apparently poorly used in polysaccharide synthesis, although there was a delayed excretion of a small amount of radioactivity (124). Since inorganic sulfate is released from the administered ester *in vivo*, it is not possible to decide whether this retention represents incorporation of the administered ester or of the inorganic sulfate that is first released from it. Enzymatic sulfation of N-acetylgalactosamine apparently leads primarily to the 4-sulfate, the position of the sulfate in chondroitin sulfate-A and -B (125). The sulfate in chondroitin sulfate-C and in chondroitin sulfate from shark cartilage are on position 6.

Sulfotransferases with specificity towards various mucopolysaccharides have been detected and studied individually. Davidson & Riley (126) have purified from rabbit skin an enzyme that is much more active with chondroitin-B than with several other similar mucopolysaccharides. The fact that only one sulfate was introduced per five to six disaccharide units, together with information available from turnover studies (127), led the authors to consider the possibility that an oligosaccharide or other low molecular weight substance may be the sulfate acceptor *in vivo*. It is noteworthy that uridine triphosphate, but not the triphosphates of guanosine, cytosine, or adenosine, stimulated the sulfation of chondroitin sulfate-B. Suzuki *et al.* (128) have separated specific mucopolysaccharide sulfotransferases by chromatography of hen oviduct extracts. The heparitin sulfotransferase was relatively free of other enzymes, but the remaining sulfotransferases were only partially separated from each other. Extracts of embryonic calf cartilage nearly free of chondroitin sulfate show sulfate transfer from 3'-phosphoadenosine-5'-phosphosulfate to both desulfo-chondroitin sulfate and to partially sulfated chondroitin sulfate (129); and a human chondrosarcoma extract, which contained chondroitin sulfate-C only, showed a specificity for it and little activity towards the A or B forms (129). Phenol and mucopolysaccharide sulfotransferase activity has also been demonstrated in beef cornea epithelial extract (130).

In an extensive investigation of the role of vitamin A in mucopolysaccharide formation, Wolf and his co-workers (131) have obtained evidence that the incorporation of sulfate is dependent upon the presence of the vitamin. The addition of vitamin A increased sulfate incorporation in rat colon segments and homogenates from vitamin A-deficient rats (132). The defect in the preparations, which were able to carry out the net synthesis of mucopolysaccharides (133), was traced to an enzyme system not specifically

concerned with the incorporation of hexose precursors (134). Finally, the defect was attributed to diminished synthesis of 3'-phosphoadenosine-5'-phosphosulfate, the condition being rectified by the addition of vitamin A to the incubation medium (135). Moretti & Wolf (136) have also reported a decrease in mucopolysaccharide-bound hexosamine (mainly galactosamine) in colon segments from vitamin A-deficient rats. These studies are of considerable interest in that they may eventually lead to the biochemical explanation for the well-known lesions in mucous membranes, more specifically in mucopolysaccharide formation, that are observed in vitamin-A deficiency.

Specificity in the desulfation of mucopolysaccharides is illustrated by an enzyme ("cellulose polysulfatase"), from a marine gastropod, *Charonia lampas*, that hydrolyzes cellulose polysulfate and charonin sulfate but has little action on chondroitin sulfate or amylose polysulfate (137).

Polymerization—depolymerization and turnover of mucopolysaccharides.—Davidson and his co-workers (138, 139) have found that incubation of chondroitin sulfate-B with a crude enzyme preparation derived from rabbit skin (or chick embryo skin) leads to the formation of a mixture of low molecular weight fragments still containing sulfate. They are investigating the interesting observation that this extensive degradation is markedly inhibited by uridine triphosphate, an agent previously found to stimulate sulfate incorporation into skin chondroitin sulfate-B (126). These effects of uridine triphosphate were not shown by other nucleoside triphosphates.

Using labeled glucose injections followed by the determination of the specific activity of hexosamines in purified connective tissue, hyaluronic acid, and mucopolysaccharides of rabbits, Davidson *et al.* (140) have observed a marked dependence of isotope incorporation on the age of the animals. In view of the fact that the acidic mucopolysaccharides of cartilage are combined with protein, Gross *et al.* (141) have studied the turnover rates of both portions of the complex by labeling the mucopolysaccharide and protein with S^{35} -sulfate and lysine-1- C^{14} , respectively. Both moieties turned over at the same rate, thus placing restrictions on speculations regarding the metabolism of the complex.

A chitinase system isolated from the hypodermis of the shore crab *Carcinus maenas* releases N-acetylglucosamine from chitin (142).

The synthesis of hyaluronic acid in cell-free extracts of embryonic skin has been accomplished by Schiller *et al.* (143) and in cell-free extracts of human synovia, embryonic cranial fibroblasts, and foreskin fibroblasts by Altschuler *et al.* (144). In both studies, UDPGA and UDP-acetylglucosamine were employed as precursors. Smith *et al.* (145) have obtained net synthesis *in vitro* of Type III pneumococcal capsular polysaccharide from UDPG and UDPGA. Characterization of the enzyme system showed that it is present in particulate fractions sedimenting between 30,000 x g and 140,000 x g and that preformed Type III capsular polysaccharide is required (146). A non-capsulated Type III pneumococcus also yields extracts capable of synthesizing the polysaccharide, although they are less active than those derived from

encapsulated organisms. The synthesis by the noncapsulated form is consistent with previous studies which demonstrated that the genetic defect involves an inability to convert UDPG to UDPGA (147). A serologically reactive polymer has been obtained by the action of a Type I pneumococcal extract on UDP-galacturonic acid (148). The fact that the single precursor sufficed for the synthesis strongly suggests that, although galactose, N-acetylglucosamine, and a methylpentose are constituents of the polysaccharide produced by the organisms (149), it may have a backbone of polygalacturonic acid.

DISACCHARIDES

Maltose.—Schramm and his co-workers (150, 151) have studied enzymatic aspects of maltose metabolism in *Neisseria perflava* which, like *Neisseria meningitides*, contains maltose phosphorylase (152). Since the enzyme splits maltose to glucose and β -glucose-1-phosphate, Ben-Zvi & Schramm (151) looked for, and found, a phosphoglucomutase specific for the reversible transformation of the β -phosphate into glucose-6-phosphate. The phosphorylase was found to be useful in the preparation of the glucosamine, N-acetylglucosamine, and 2-deoxyglucose analogues of maltose (150). Certain lactobacilli from beer that grow better on maltose than on glucose have also been shown to contain maltose phosphorylase (153).

Sucrose.—Sucrose synthesis in plants is being investigated further. Bean (154) found that soluble preparations from the juice of mature oranges, but only particulate preparations from lemon juice, catalyzed the synthesis of sucrose from UDPG and fructose. There was no evidence for the system utilizing UDPG and fructose-6-phosphate for the formation of sucrose phosphate. However, Calvin & Beevers (155) interpret their isotope experiments on slices of endosperm from castor bean seedlings as favoring the second pathway. The sucrose-synthesizing systems of wheat germ and spinach leaves have been studied by Mendicino (156), who purified the enzyme catalyzing the synthesis of sucrose-6-phosphate from the one producing free sucrose directly. From several lines of evidence, it was concluded that the pathway including sucrose-6-phosphate is probably the more important one for sucrose biosynthesis.

Lactose.—The mechanism of formation of lactose has proved to be a more difficult task because of complications in the appraisal of tracer data. However, Watkins & Hassid (157) have now isolated a particulate fraction from lactating guinea pig mammary gland which catalyzes the synthesis of lactose from UDP-galactose and glucose. No evidence was found for the intermediate formation of lactose-1-phosphate, which is formed from UDP-galactose and glucose-1-phosphate and then hydrolyzed to lactose in the enzymatic system extracted by Gander *et al.* (158) from lactating cow mammary gland. Although the Watkins-Hassid synthetic mechanism is consistent with the tracer studies of Wood *et al.* (159), further evaluation of the two mechanisms for lactose production appears necessary. Malpress

(160) has observed correlation of the activities of phosphoglucomutase, inorganic pyrophosphatase, and UDPG pyrophosphorylase in homogenates of rat mammary glands with the milk secretory activity of the glands at different stages of lactation.

Trehalose.—A detailed enzymatic study, by Candy & Kilby (161), of the locust fat body has yielded evidence for the existence of the trehalose-synthesizing system originally discovered in yeast by Cabib & Leloir (162), namely, the production of trehalose phosphate from UDPG and glucose-6-phosphate, followed by the action of a specific trehalose phosphatase. Tracer studies indicate that trehalose metabolism in silkworm pupae is markedly affected by superficial injury, which had previously been shown to cause metabolic changes resembling those that accompany hormone-induced adult development (163).

HEXOSE METABOLISM VIA THE EMBDEN-MEYERHOF PATHWAY AND RELATED TOPICS

Glucose metabolism via the Embden-Meyerhof pathway.—The rate of metabolism of glucose by a tissue is dependent upon its rate of entry into cells and its phosphorylation to glucose-6-phosphate. Since Sols (164) has so fully discussed the transport of sugars, this subject will not be extensively reviewed here except when studies on transport are quite relevant to the subject under discussion. Reports which may be consulted deal with the following subjects: a review on intestinal transport (165), a suggested carrier mechanism for the intestinal transport of D-xylose (166), a study of inhibitors of xylose permeability in rat diaphragm muscle (167), the formation of galactose-1-phosphate in rat intestine during galactose absorption (168), and the absorption of various sugars and sugar derivatives in strips of hamster intestine (169, 170). In addition, sulfhydryl inhibitors and uncouplers of oxidative phosphorylation have been found to stimulate the transport only of those sugar isomers which are sensitive to insulin (171), and the effect of insulin on sugar transport in the isolated perfused rat heart has been shown to be inhibited by N-ethylmaleimide, a sulfhydryl blocking agent (172). Kinetic aspects of the effect of insulin on glucose penetration and phosphorylation in frog sartorius muscle have been studied by Narahara *et al.* (173) and the results discussed in terms of hypothetical models of glucose transport across membranes.

It is often difficult to distinguish the two processes of transport and phosphorylation in studies on glucose utilization. In an extensive series of experiments on the isolated, perfused heart of the rat, using techniques allowing differentiation of the two processes, Morgan, Park, and their colleagues (174 to 180) have investigated various physiological influences on the glucose uptake of the hearts of normal, diabetic, and hypophysectomized animals. Kinetic analysis indicated that the processes of transport and phosphorylation can each be described by Michaelis-Menten kinetics (176). In general, transport, rather than phosphorylation, is the major limiting step

in the utilization of this sugar. Stimulation of transport by insulin can make phosphorylation the major limiting process. In the diabetic heart, although insulin restores transport to the normal rate, glucose uptake does not become normal, because glucose phosphorylation is depressed. This depression is attributed to the action of hormones of the pituitary and adrenal glands, as the inhibition is removed by hypophysectomy or adrenalectomy. Moreover, injection of both growth hormone and hydrocortisone to the hypophysectomized-adrenalectomized animal reduces phosphorylation to 15 per cent of normal (179). Kipnis & Cori (181) similarly found that the intact diaphragm from the diabetic rat exhibits subnormal phosphorylation as well as an insulin-insensitive defect in transport. The phosphorylation capacity was considered to be under the control of the pituitary-corticoadrenal system.

In normal and diabetic hearts, anoxia accelerates both transport and phosphorylation (174, 175). By determining the concentrations of glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate, Newsholme & Randle (182) obtained evidence that anoxia accelerates the phosphorylation of fructose-6-phosphate, thereby lowering the concentration of glucose-6-phosphate, an inhibitor of hexokinase. Similarly, Threlfall & Stoner (183) found that hindlimb ischemia leads to elevation of fructose diphosphate and pyruvate in the forelimb. As might be expected from these results, in a situation where phosphorylation is inhibited, Regen *et al.* (184) have observed that the steady-state levels of glucose-6-phosphate and fructose-6-phosphate in the perfused heart of the diabetic rat are almost twice the normal levels. The soluble, hepatic glucokinase of the alloxan-diabetic rat was found by DiPietro & Weinhouse (185) to be depressed to about 40 per cent of the normal level, this decrease being quite moderate relative to the generally poor utilization of glucose by liver slices of alloxan-diabetic rats (186).

The existence of tissue specificity in the action of hormones and other agents on glucose utilization is well known. The increased glucose uptake by rat adipose tissue treated with adrenocorticotrophic and growth hormones has been found to be accompanied by an increase in the glycolytic pathway but not in the phosphogluconate route (187). Unlike the effect of alloxan-diabetes on glucose transport in heart muscle, active transport of sugars by the small intestine is increased in the diabetic animal. In experiments designed to isolate the transport phenomenon, Crane (188) has used, in experiments *in vitro*, 6-deoxy-D-glucose, which is actively transported but not metabolized, and galactose, which is also actively transported but metabolized more slowly than glucose. The elevated rate of sugar transport shown by the alloxan-diabetic rat was not decreased appreciably by the addition of insulin to the preparations or by pretreatment of the animals with the hormone. 6-Deoxy-D-glucose was shown by Brooks *et al.* (189) to inhibit glucose oxidation in rat kidney slices, mouse adipose tissue, and rat diaphragm, the site of the competitive inhibition being presumed to be in the membrane transport or the phosphorylation processes. 6-Deoxyglucose labeled with C^{14} did not yield any $C^{14}O_2$ with mouse adipose tissue.

2-Deoxyglucose inhibition of glucose utilization may be more complicated than heretofore assumed. Barban & Schulze (190) found that human cell cultures grown on fructose are more sensitive to the inhibitor than those grown on glucose and that 2-deoxyglucose-6-phosphate inhibits fructokinase, phosphohexose isomerase, and glucose-6-phosphate dehydrogenase from HeLa cells. The deoxyglucose derivative also was itself slowly oxidized by glucose-6-phosphate dehydrogenase. A strain of HeLa cells resistant to 2-deoxyglucose has been found to contain a nondialyzable, heat-labile inhibitor of the analogue (191). A related problem that requires explanation is the mechanism by which 2-deoxyglucose inhibits the Crabtree effect in ascites carcinoma cells (192). In man, 2-deoxyglucose increases plasma lactate markedly (193) although in tumor cells the net production of lactate decreases (192). 2-Deoxyglucose-6-phosphate competitively inhibits the stimulation of glycogen synthetase by the 6-phosphates of glucose, fructose, and glucosamine (15).

It is interesting that skin maintained on 2-deoxy-D-glucose not only produces 2-deoxyglucose-6-phosphate (194) but a substance that appears to be identical to synthetic D-mannonic acid-6-phosphate (195). If, as Brooks *et al.* (195) suggest, the latter substance is derived from 2-deoxy-D-gluconic acid-6-phosphate that is probably formed initially, then a novel reaction must exist for converting a methylene group to an alcohol group in a sugar.

Mitochondrial glycolysis, already reported to occur in the brain and in certain tumors (196), has now been described by Étingof & Krichevskaya (197) in mitochondria of cultured cells of the monkey heart. Aisenberg (198) has critically discussed the question of mitochondrial glycolysis.

The complexity of the mitochondrial stimulation of glycolysis ("negative Pasteur effect") was indicated by the study of Schwartz & Lee (199), who showed that heart mitochondria (unlike liver mitochondria) stimulate glycolysis of brain or heart supernatant. The effect was observable with mitochondria from normal guinea pigs and also from animals in which experimental congestive heart failure had been produced. In the latter case, the severe uncoupling with respect to oxidative phosphorylation did not interfere with ability to influence glycolysis. The Pasteur effect of α -ketoglutarate in aerobically glycolyzing homogenates of ascites tumor cells was attributed by Emmelot & Bos (200) to the mediation of oxidative phosphorylation and to an inhibition of hexokinase. Tiedemann & Born (201) also invoke oxidative phosphorylation, the availability of orthophosphate, and the inhibition of hexokinase in explaining the Pasteur effect, whereas Sachsenmaier (202) believes the agent is the nicotinamide-adenine dinucleotide of liver particles. The effect of certain ions in reversing the Pasteur effect in rat brain mitochondria has been studied by Rendina & Hellerman (203).

The Crabtree effect has been studied in connection with the effect of 2-deoxyglucose on the citric acid cycle (204), and key roles of nicotinamide-adenine dinucleotide phosphate reduction and the turnover rate of glucose

via the hexose monophosphate shunt have been proposed as an explanation of the phenomenon (205). Electron microscopic examination of ascites tumor cells has disclosed certain mitochondrial changes accompanying the Crabtree effect (206, 207). Short-term metabolic alterations induced by the addition of glucose to these cells have been determined (208). For critical discussions of the Pasteur and Crabtree effects, the recent reviews by Aisenberg (198), Ibsen (209), and van Eys (210) may be consulted.

In a study designed to test Warburg's hypothesis that tumor cells derive much of their energy from glycolysis, Papaconstantinou & Colowick (211, 212) have investigated the effect of oxamate, a pyruvic acid analogue that competitively inhibits lactic dehydrogenase, on the metabolism of Ehrlich ascites tumor cells and on the growth of HeLa cells in tissue culture. The analogue inhibited aerobic and anaerobic glycolysis of the ascites tumor cells, which, however, were much less sensitive to oxamate than was lactic dehydrogenase isolated from these cells. Moreover, under anaerobic conditions, the accumulation of pyruvate eventually reversed the inhibition; aerobically there was no pyruvate accumulation or reversal of inhibition. Similarly, oxamate inhibited growth, glucose utilization, and lactate production of HeLa cells, and its effects were reversed by pyruvate. Since only an effect on lactic dehydrogenase could be detected, it was concluded that this enzyme is essential for growth of the cells. The very rapid excretion of oxamate by animals discouraged tests of the inhibitor on glycolysis *in vivo*.

The observation that pyruvate stimulates the oxidation of radioactive glucose or lactate to carbon dioxide in ascites tumor cells has been further investigated by Wenner & Paigen (213), who found that the effect of pyruvate can be explained by its serving as an additional electron acceptor, a conclusion rendering postulations of unknown metabolic pathways for pyruvate, or compartmentalization of exogenous and endogenous pyruvate, unnecessary.

Siu *et al.* (214) have discovered a new enzyme which catalyzes the formation of oxaloacetate from phosphoenolpyruvate. The enzyme, the third known to effect this transformation, was obtained from *Propionobacterium shermanii* and has been named phosphoenolpyruvic carboxytransphosphorylase. The reaction of phosphoenolpyruvate, carbon dioxide, and phosphate reversibly yields oxaloacetate and pyrophosphate. The authors suggest that the carbon dioxide fixed in the reaction should occur in propionate as a result of the pathway involving succinate and methylmalonic acid derivatives. The endogenous formation of phosphoenolpyruvate from inorganic phosphate by rat liver mitochondria was found by Schellenberg & Weinbach (215) to be enhanced by 2,4-dinitrophenol and pentachlorophenol but not by other uncouplers of oxidative phosphorylation. In a study of the dynamics of blood glucose production and utilization (216, 217), the data indicated that glucose resynthesis occurs in the rat via the "dicarboxylic acid shuttle" and that this pathway is activated by starvation (217).

As an aspect of a study of thiamine deficiency which originated from the

discovery that a thiazole derivative, thiamic acid, is associated with crystalline NAD-linked rabbit muscle α -glycerophosphate dehydrogenase (218), van Eys (219) has now observed that thiamine-deficient rats have greatly depressed levels of α -glycerophosphate dehydrogenase in liver and muscle. Oxythiamine and pyrithiamine also lower the tissue levels of this enzyme. A decrease in lactic dehydrogenase is simultaneously induced, but the analogues and simple dietary thiamine deficiency show differential effects on the two enzymes.

Employing newly developed histochemical methods for enzymes in skeletal muscle, Pearse (220) has found that mitochondrial α -glycerophosphate dehydrogenase shows the highest activity in the group of larger fibers, which are high in phosphorylase activity but low in oxidative enzymes. Since the nonmitochondrial α -glycerophosphate occurred predominantly in the smaller fibers, the observations were considered not to lend support to the concept of an α -glycerophosphate cycle as an important pathway in the larger fibers. Ciacio & Keller (221) have presented evidence that hamster liver mitochondria contain both the NAD-linked and flavo-protein α -glycerophosphate oxidizing enzymes.

Chang *et al.* (222) have reported that vitamin B₁₂ deprivation indirectly elevates the ratio of NAD to its reduced form in rat liver and causes accumulation of blood pyruvic and lactic acids. Several studies concerned with alterations in tissue levels of carbohydrate-metabolizing enzymes will be discussed in the section on the hexose monophosphate shunt.

Siebert (223) has found that isolated cell nuclei from rat liver and pig kidney contain all the enzymes, substrates, and cofactors required for the glycolytic pathway. Evidence for a functional glycolytic system in the H37Ra strain of *Mycobacterium tuberculosis* has been presented by Bastarrachea *et al.* (224), and McDonald *et al.* (225) have shown with specifically labeled glucose that a combined glycolysis-Krebs cycle pathway accounts for over 90 per cent of glucose catabolism in *Claviceps purpurea* (ergot fungus).

Since nicotinamide-adenine dinucleotide phosphate-linked glyceraldehyde phosphate dehydrogenase has previously only been found in leaves, it has been assumed that the enzyme is linked to this nicotinamide-adenine phosphate because of the role of this coenzyme in photosynthesis. Benedict & Beevers (226) have now reported finding nicotinamide-adenine dinucleotide phosphate-linked glyceraldehyde phosphate dehydrogenase activity in non-photosynthetic plant tissue, the endosperm tissue of castor beans grown in the dark. In a further study on the asymmetric labeling of starch from C¹⁴O₂, Kindel & Gibbs (227), using *Chlorella pyrenoidosa*, found that light is not necessary for this asymmetric labeling. Asymmetric labeling of starch from acetate was attributed to a slowly operating carbon cycle superimposed on the more rapid tricarboxylic acid-glycolytic route. The isolation of sulfolactaldehyde and related observations led Benson & Shibuya (228) to consider the possible existence of a sulfoglycolytic sequence in plant metabolism.

Fructose.—Two ketokinases have been found in homogenates of intestinal mucosa by Cadenas & Sols (229). Since both enzymes phosphorylate D-fructose (and L-sorbose) in the 1-position, their activities are consistent with the initial reaction of the pathway for the conversion of fructose to glucose in liver. In this organ, fructose-1-phosphate is cleaved, through the action of aldolase, into dihydroxyacetone phosphate and glyceraldehyde, the latter being converted directly to 3-phosphoglyceraldehyde, to glycerol, or to glyceric acid. Heinz & Lamprecht (230) have now confirmed the original report of Hers & Kusaka (231) on the occurrence of a liver enzyme, triose-kinase, which, with adenosine triphosphate as phosphate donor, catalyzes the formation of 3-phosphoglyceraldehyde from glyceraldehyde. Dihydroxyacetone will also serve as a substrate, but glycerol, D-glyceric acid, glycolaldehyde, glucose, and fructose are inactive. However, Kattermann *et al.* (232) have obtained physiological evidence indicating that the glycerate pathway is important in fructose metabolism. The concentration of D-glycerate in rat liver was increased greatly by intraperitoneal injections of fructose or by direct infusion of isotonic fructose by the vena portae.

There is now additional evidence that fetal liver cannot utilize fructose. In 1957 Hers (233) had reported an inability to demonstrate fructokinase in this tissue. Andrews *et al.* (234) now find that fetal liver, as well as neonatal liver before the fifth day of birth, does not utilize fructose in perfusion fluid.

The sorbitol mechanism for the conversion of glucose to fructose (235, 236), while generally accepted as the basis for the formation of seminal fructose, has had to share attention with the mechanism involving the placental hydrolysis of a fructose phosphate for the formation of fetal fructose. Perfusion experiments with goat placenta have not resolved the problem (237).

Mannose.—Although many aspects of mannose metabolism have been studied in the past, there have been few complete investigations of its metabolic reactions in a single tissue. Abraham *et al.* (238) have now shown that mannose-1-C¹⁴ is well utilized by slices of the lactating rat mammary gland, yielding C¹⁴O₂ and labeled fatty acids as efficiently as those obtained with glucose-1-C¹⁴. Since the hexoses were equally effective in promoting the conversion of acetate to fatty acids, the authors postulated that mannose is rapidly converted to glucose-6-phosphate, thus providing a mechanism for NADPH₂ generation via the hexose monophosphate shunt. Evidence was then obtained for the existence of mannokinase (which presumably yields mannose-6-phosphate), phosphomannose isomerase (for the production of fructose-6-phosphate), and phosphoglucomutase. Wood *et al.* (239) have found that the metabolism of mannose-C¹⁴ is similar to that of glucose in rat epididymal tissue and in rat liver slices *in vitro*, although the rate of mannose utilization is about 60 to 90 per cent that of glucose. The utilization of the two hexoses was also comparable in diabetic tissue, and insulin affected them similarly.

Galactose.—In order to rule out the possibility that the conversion of

galactose to glucose may involve a bound inositol intermediate, Kohn & Dmuchowski (240) determined the labeling in the liver glycogen of the rat following the administration of D-galactose-2-C¹⁴. The finding that the bulk of the isotope was in position C-2 of glucose precluded an inositol route from galactose to glucose and was in accord with the accepted pathway for the transformation. The inhibition of galactose metabolism by the administration of ethanol has been investigated by Isselbacher & Krane (241), who found that the alcohol leads to the production of reduced diphosphopyridine nucleotide, an inhibitor of UDP-galactose-4-epimerase. This work illustrates the sensitivity of galactose metabolism to alterations in the ratio of NAD to its reduced form.

Although the enzymatic mechanism for the conversion of galactose to glucose is established, many interesting problems remain in relation to galactose toxicity and hereditary defects in galactose metabolism in both man and microbes. Lerman (242, 243) has suggested that the depressed activity of glucose-6-phosphate dehydrogenase in galactosemic cataracts of rats may be caused by galactose-1-phosphate, the intermediate that accumulates in galactosemic patients. Evidence not in harmony with this hypothesis was obtained by Weinberg & Segal (244), who showed that when galactosemic leucocytes accumulate abnormally high amounts of galactose-1-phosphate, there is no inhibition of glucose metabolism via the hexose monophosphate pathway. Similarly, Korc (245) found that incubation of normal lens homogenate with galactose-1-phosphate does not affect the activity of the glucose-6-phosphate dehydrogenase. Rosenberg *et al.* (246) have based another test of the Lerman hypothesis on the fact that long-term feeding of high galactose diets to rats leads to renal tubular dysfunction, polyuria, cataract, and other symptoms similar to those accompanying galactosemia. The proportion of glucose metabolized via the shunt in kidney slices of the galactose-fed rats did not differ significantly from normal values.

Nikaido (247) has continued his studies on mutants of *Salmonella* (M mutants) that have a specific hereditary defect in UDP-galactose-4-epimerase. They form cell walls composed only of glucose, whereas the wild type of cell walls contains galactose, mannose, rhamnose, and tyvelose as well as glucose. Since the enzymatic defect in the production of the epimerase is presumably the only alteration in enzyme production resulting from the single gene mutation, the marked alteration in sugar composition of the cell wall was a matter for speculation. The organisms undergo severe bacteriolysis when grown in the presence of galactose, although, as expected, secondary mutants lacking galactokinase are resistant to this sugar. The lysis of the epimerase-deficient cells was demonstrable only with growing cells (248). The possible mechanisms whereby the defective galactose metabolism induces the lytic phenomenon were discussed in detail. A similar epimerase-deficient organism, *E. coli* C7M, grown in stationary phase in the presence of galactose, has been used by Wiesmeyer & Jordan (249) to develop a simple procedure for the preparation of UDP-galactose. Kalckar (250) has recently

reviewed hereditary defects in galactose metabolism in man and in microorganisms.

Galactose-1-phosphate uridyl transferase, previously not detected in plants, has been found in soybean extracts by Pazur & Shadaksharaswamy (251).

Two other findings regarding galactose metabolism may be relevant. Avigad *et al.* (252) have discovered a new type of enzymatic system in the wood mold *Polyporus circinatus*, which contains a galactose oxidase (253) catalyzing the oxidation of the C-6 position of galactose (yielding galactose-dialdose) as well as of galactosides. Guanosine diphosphate-L-galactose has been isolated by Su & Hassid (92) from a strain of marine red alga, *Porphyra perforata*, an organism containing a polysaccharide consisting of both D- and L-galactose.

Other studies on aldohexose kinases.—Substrate specificity studies on kinases from various sources have been carried out: galactokinase from *Saccharomyces fragilis* (254), hexokinase from *Aspergillus oryzae* (255), glucokinase and N-acetylglucosaminekinase from *E. coli* (256), and hexokinase preparations from rat intestine, kidney, and liver (257). This last investigation employed several rare aldoses, the utilization of which for CO₂ and glycogen production was compared in a related study (258).

A new type of enzymatic phosphorylation has been discovered by Smith (259, 260). Extracts of succinate-grown *E. coli* catalyze a direct phosphorylation of glucose with potassium phosphoramidate, with N-phosphorylglycine, or, more slowly, with monophosphorylhistidine but not with creatine phosphate or phosphordiamidate. The product is glucose-1-phosphate.

THE HEXOSE MONOPHOSPHATE (PENTOSE PHOSPHATE) PATHWAY AND PENTOSE METABOLISM

Enzymes and intermediates of the hexose monophosphate pathway.—Much of the recent work on this pathway has been concerned with the evaluation of its physiological significance, but there has also been continued progress in the more enzymological aspects. Julian, Wolfe & Reithel (260a) have achieved the crystallization of glucose-6-phosphate dehydrogenase of bovine mammary gland, and Noltmann, Gubler & Kuby (261) are the first to accomplish the crystallization of this enzyme from brewer's yeast. The latter investigators described an efficient large-scale process involving several uncommon operations, including the use of NADP, the coenzyme of the dehydrogenase, to induce crystallization.

Two laboratories (262, 263) have observed rather striking inhibitory effects of certain steroids on glucose-6-phosphate dehydrogenase. Marks & Banks (263) found that dehydroisoandrosterone, pregnenolone, and certain related steroids, at concentrations of 1×10^{-6} M or less, inhibit mammalian glucose-6-phosphate dehydrogenase but not the yeast or spinach enzyme. Moreover, the steroids did not inhibit 6-phosphogluconate dehydrogenase or isocitric dehydrogenase of mammals. Since McKerns & Bell (262) obtained

somewhat different results, for example, in the structural requirements of the steroids, further investigation of these effects is required.

Work on glucose-6-phosphate dehydrogenase in human red cells is continuing because of its key role in certain genetic abnormalities (264). Marks *et al.* (265) have found that, although the enzymes from normal subjects and from Negroes with a genetically determined deficiency of this enzyme appear to be similar in various physical and chemical properties, the preparations from normal human beings show a greater degree of activation upon incubation at 37° than do the extracts from the deficient individuals. Ramot *et al.* (266) have continued their interesting study of the activation of the dehydrogenase of enzyme-deficient subjects. They present evidence that normal erythrocyte stroma contains an activator that is responsible for the amount of active dehydrogenase found in extracts of erythrocytes. Since the stroma factor, which appears to have the properties of an enzyme, activates preparations from deficient individuals but not from normal subjects, and since the activator was not found in the deficient subjects, it was suggested that the genetic lesion may affect the synthesis of an activator of an inactive form of the enzyme, rather than the synthesis of the enzyme protein itself. The relationship of these two activation studies (265, 266) is not clear at present. The mechanism of pentose phosphate synthesis has been studied in human beings with a deficiency in the oxidative pathway for glucose-6-phosphate metabolism. In favism red blood cells, Bonsignore *et al.* (267) have found elevated levels of transketolase and transaldolase, which may represent a compensatory mechanism for pentose phosphate synthesis.

In an investigation of crystalline transketolase of yeast, Datta & Racker (268) have obtained evidence for multiple attachments of thiamine pyrophosphate to the enzyme and have observed that oxythiamine pyrophosphate, a potent inhibitor of the enzyme, has a much greater affinity for the apoenzyme than does thiamine pyrophosphate. Transketolase-catalyzed exchange reactions between glycolaldehyde and fructose-6-phosphate led to the postulation of a weakly dissociable glycolaldehyde-enzyme intermediate and then to the isolation of the substrate-enzyme intermediate (269), which is presumed to contain dihydroxyethyl thiamine pyrophosphate, a substance recently synthesized by Krampitz (270). In a preliminary note, Bradbeer & Racker (271) have reported that the active glycolaldehyde-transketolase intermediate yields glycolate in the presence of ferricyanide or spinach chloroplasts. In the latter system, but not in the former, the reaction was stimulated by light.

Ljungdahl *et al.* (272) have shown that transaldolase, which forms a dihydroxyacetone-enzyme intermediate (273), catalyzes an exchange between glyceraldehyde-3-phosphate and fructose-6-phosphate. This exchange is of interest because it may be the explanation for C¹⁴ patterns obtained in metabolic experiments, where unequal labeling of positions C-3 and C-4 of hexoses formed from 3-carbon precursors has frequently been observed instead of the expected equal labeling of these positions. Moreover, the

transfer reaction involving glyceraldehyde-3-phosphate and fructose-6-phosphate provides a convenient method for the preparation of glucose-4-C¹⁴ or glucose-5-C¹⁴ when suitably labeled triose phosphate is employed as substrate.

Bonsignore *et al.* (274) have identified as sedoheptulose-7-phosphate the heptulose phosphate formed by the action of rat liver supernatant fraction on hexose monophosphate. This rigorous characterization was made necessary by the report of Nigam *et al.* (275) on the production, in a similar system, of a new 7-carbon metabolite that was tentatively characterized as a 3-keto-D-araboheptose-7-phosphate. Sie *et al.* (276) interpret their enzymatic results as indicating the existence of two types of heptulose phosphate-forming systems in addition to the well-established transketolase-catalyzed production of sedoheptulose-7-phosphate from pentose-5-phosphates. One of the new systems converts nucleosides and inorganic phosphate to sedoheptulose monophosphate (277), while the other utilizes certain hexose phosphates for the production of 3-keto-D-araboheptose-7-phosphate (275, 278). Differences in the sensitivity to thiamine deficiency of these systems in different tissues have been reported (276), and their distribution in nature has been studied (279).

Sedoheptulose diphosphatase, which has not been found in animal tissues, has been used in a reconstructed enzyme system capable of dephosphorylating fructose-1,6-diphosphate to fructose-6-phosphate (280). Sedoheptulose diphosphate accumulates in significant amounts during the metabolism of ribose by the intact red blood cell (281). The role of this diphosphate in mammalian metabolism is unknown at present.

Dische & Igals (282) have presented evidence for the formation of unidentified substances in human hemolysates incubated with certain sugar phosphate intermediates. For example, erythrose-4-phosphate yields a substance that does not respond to color reactions of tetroses, pentoses, hexoses, heptoses, or octoses. The new substances are formed in hemolysates containing residual aldolase, that is, aldolase activity which is more resistant to incubation at 43° at pH 8.4 than the total transaldolase originally present (283). The inhibitory effects of erythrose-4-phosphate on transketolase and glucose-6-phosphate isomerase are discussed in terms of a feedback mechanism (282). It is to be hoped that identification of the new intermediates and the elucidation of the inhibitory mechanisms can readily be accomplished.

Contribution of the hexose monophosphate pathway in glucose oxidation.—The contribution of the hexose monophosphate shunt has been evaluated in a number of organisms. Through the use of specifically labeled glucose, Segal *et al.* (284) have estimated that about 8 per cent of glucose is metabolized by this pathway in man. Evidence for this metabolic route has also been obtained in slices of skin from young rats (285), in slices of calf anterior pituitary (286), in islet and ascinar tissue of rabbit pancreas (287), in skin and feather primordia of the developing chick embryo (288), and in cartilage

slices of weanling rachitic rats (289). The pathway is believed by Rahman & Kerly (290) to be responsible for oxidation of one-fourth of the glucose utilized *in vitro* by ox retina. A comprehensive study of the metabolism of glucose carbon atoms by tobacco leaf disks indicated a significant contribution of the pentose cycle (291). Although sonic extracts of *Erwinia amylovora* were shown to contain the enzymes necessary for the operation of the shunt, fermentation experiments with resting cells of this organism pointed to the operation of the Embden-Meyerhof pathway rather than the hexose monophosphate or Entner-Doudoroff pathways (292). The hexose monophosphate pathway is reported to be the major route in *Acetobacter melanogenum* and *industrium* (293), and in both growing and resting cells of *Pasteurella multocida* (294). Culture of *E. coli* at pH 5 instead of at more alkaline pH leads to the oxidation of a limited portion of glucose via the phosphoglucuronate pathway (295). The work of Stouthamer (296) indicates that *Gluconobacter liquefaciens* metabolizes gluconate by this pathway after phosphorylation.

The relative contribution of the glycolytic and the hexose monophosphate oxidation pathways for glucose oxidation is dependent on many factors. In a study of the utilization of tritium from glucose-1- H^3 and lactate-2- H^3 in fatty acid synthesis, Lowenstein (297) has obtained data indicating that the direct oxidation of glucose-6-phosphate is not a major source of NADPH₂ in the livers of rats on normal diets; and even during hyperlipogenesis, when glucose-6-phosphate dehydrogenase is markedly elevated, the contribution of glucose-1- H^3 was less than that of lactate-2- H^3 . However, the relative contributions are reversed in the lactating mammary gland.

Additional evidence has been obtained for a controlling role of pyridine nucleotides. Butt & Beevers (298) have observed that, in maize roots, the operation of the shunt is apparently limited severely by the supply of oxidized nicotinamide-adenine dinucleotide phosphate which can be increased by the addition of nitrite. A similar conclusion was reached by Dumont (299) in a study on the utilization by thyroid tissue of specifically labeled glucose. The addition of Synkavit, an artificial electron acceptor, or of mono- or diiodotyrosine, substrates of an NADPH₂-linked thyroid enzyme, enhances the oxidation at position C-1 of glucose. Field *et al.* (300) have shown that the ability of physiological concentrations of thyroid-stimulating hormone to enhance glucose oxidation by thyroid slices can be explained by an increase in nicotinamide-adenine dinucleotide phosphate concentration in the tissue. The stimulation *in vitro* of glucose oxidation in the thyroid by acetylcholine and by thyroid-stimulating hormone involves different mechanisms, as the latter stimulates glucose-1-C¹⁴ oxidation more than glucose-6-C¹⁴ oxidation, whereas the former stimulates both equally (301). Unlike growth hormone and ACTH in their effects on glucose oxidation in the lactating mammary gland of the rat, prolactin specifically stimulates glucose-1-C¹⁴ oxidation but does not affect glucose-6-C¹⁴ oxidation (302). The addition of serum or yeast extract, presumably by increasing coenzyme con-

centrations, stimulates the phosphogluconate pathway in the intact thoracic aorta of guinea pigs (303). Seelich & Letnansky (205) have proposed that the dependence of the Crabtree effect on glucose concentration and phosphate content of the medium is a result of the turnover of glucose via the shunt.

Using tissues from thiamine-deficient rats, Bagchi (304) has observed an increased oxidation of glucose-1-C¹⁴ to C¹⁴O₂ relative to that of glucose-6-C¹⁴. Although no information was obtained concerning the actual extent of oxidation via the glycolytic pathway or hexose monophosphate shunt, the alteration towards a relatively greater participation of the latter pathway may reflect its ability to oxidize glucose without a need for thiamine pyrophosphate-requiring enzymes. The effect was demonstrable in liver and lung but not in brain or kidney, organs in which the shunt pathway is not significant (305).

Regardless of whether insulin was present in the incubation medium (306), the significant contribution of the phosphogluconate pathway in mouse adipose tissue did not vary appreciably among normal mice, the obese-hyperglycemic strain of mice, and mice suffering from obesity induced by gold thioglucose. Tombropoulos & Kleiber (307) have found that, in bovine ketosis, there is a decreased participation of the shunt which can be correlated with the impaired lipogenesis in the ketotic cows.

The effect of adrenocorticotrophic hormone on glucose-6-phosphate and 6-phosphogluconate dehydrogenases in rat adrenal tissue has been studied histochemically by Greenberg & Glick (308). Tepperman *et al.* (309) have reported that triiodothyronine injected into hypophysectomized rats increases the depressed level of hepatic glucose-6-phosphate dehydrogenase to above normal level. However, much more striking increases in the enzyme level (in both uninjected and hormone-treated animals) resulted from feeding a high fructose, low fat diet for three days. Marked changes have been observed in the hepatic level of this dehydrogenase in fasting and refeeding experiments on chickens (310). Alterations in levels of shunt and glycolytic enzymes have also been determined in rats with fatty degeneration of the liver (311), in embryonic and young rats (312), and in scorbutic guinea pigs (313). Differential effects of refeeding and of cortisone administration on the production of various enzymes in rat liver have been demonstrated by Weber *et al.* (314). Ethionine was not uniformly effective in blocking the increase in enzyme levels.

In a continuation of a study of the enzymes in various tracts of the central nervous system, McDougall *et al.* (315) have found that heavily myelinated tracts, which are relatively high in lipid, have much larger amounts of glucose-6-phosphate dehydrogenase than lightly myelinated ones, but a corresponding difference in 6-phosphogluconate dehydrogenase was not observed. The data were discussed in relation to O₂ uptake, lipid and NADPH₂ metabolism, and anatomical considerations. Tracer experiments by Noble *et al.* (316) have indicated that in rabbit polymorphonuclear

leukocytes there is incorporation of carbon dioxide by a slow reversal of the 6-phosphogluconate oxidation reaction. In leukocytes, D-ribose-1-C¹⁴ and D-xylose-1-C¹⁴ yield glycogen with very high ratios of C¹⁴ in positions C-1 to C-3, the data suggesting that these ratios result from a transketolase-catalyzed exchange reaction accompanying slow net synthesis of glycogen via the transketolase-transaldolase sequence (317).

A tendency towards formation of ketopentoses from ribose-5-phosphate has been reported to occur in the serum of patients with malignant neoplasias, especially advanced metastatic cancer and acute leukemia; this tendency is greater than that found in normal individuals or those with non-malignant diseases (318).

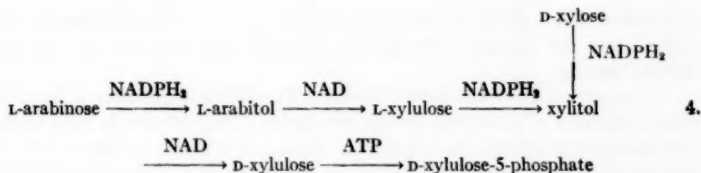
Biosynthesis of ribose and deoxyribose.—To explain certain discrepancies in previous studies of pentose synthesis in *E. coli*, Szynekiewicz *et al.* (319) have examined *E. coli* cultures grown under a variety of conditions. Comparing ribose synthesis via the oxidative route (from 6-phosphogluconate) and from the transketolase-transaldolase (nonoxidative) route, they found that the latter pathway predominates when acetate is the principal source of carbon and when acetate-grown cells are in the resting or stationary phases in glucose medium. However, the oxidative pathway predominates when the organisms are growing logarithmically in glucose medium. In *Alcaligenes faecalis* grown on acetate-1-C¹⁴, Brennenman *et al.* (320) have obtained evidence for synthesis of ribose from the condensation of 2- and 3-carbon compounds, a mechanism that has received little support in recent years. Enzymatic studies indicated the existence of the tricarboxylic and glyoxylate cycles and the transketolase-transaldolase pathway but not of the oxidative route from hexose phosphate. The labeling pattern in the RNA-ribose could be explained by the fact that the C₂+C₃ condensation is faster than the transketolase-transaldolase-catalyzed mechanism.

Wright *et al.* (321) studied the synthesis of deoxyribose in bacteriophage-infected *E. coli* by comparing labeling patterns in phage DNA-deoxyribose and in phage DNA-glucose which is bound glycosidically to hydroxymethylcytosine. Approximately 60 per cent of the pentose appeared to have been synthesized by the 6-phosphogluconate oxidation route, and no support was obtained for a C₂+C₃ mechanism. Phage infection did not appear to alter the relative contributions of the oxidative and nonoxidative routes. Cohen *et al.* (322) have obtained more specific information regarding the molecular level at which ribose is reduced to deoxyribose, a transformation for which there is now abundant evidence. *E. coli* extracts containing phage (T6r⁺)-induced RNA accumulated mainly acid-soluble 5'-ribonucleotides derived from the phage RNA. In the presence of exogenous NADPH₂, these ribonucleotides decreased in amount and deoxyribonucleotides were formed. Further studies on the NADPH₂-linked reduction of the ribonucleotides to the deoxyribonucleotides will be required to establish the level of phosphorylation of the ribonucleotide substrates.

Pentose-pentitol interrelationships.—In animals xylitol is known to be an intermediate in the glucuronate-xylulose cycle (323), and arabitol has been

found in human urine (324). McCormick & Touster (325) have studied the conversion of ribitol, D-arabitol, and L-arabitol to liver glycogen in the rat and guinea pig. Since ribitol-1-C¹⁴ labeled the glycogen chains predominantly in positions C-1 and C-3, it appears likely that this pentitol is metabolized via the pentose phosphate pathway after its initial conversion to D-ribulose. L-Arabitol-1-C¹⁴ gave a glycogen-labeling pattern suggesting extensive chain cleavage of the pentitol before it enters known metabolic pathways. Very little of the label in D-arabitol-1-C¹⁴ was found in liver glycogen.

Metabolic and enzymatic studies have recently disclosed that pentoses may be reduced to pentitols in the initial phase of their utilization by the filamentous fungi and yeasts. As elucidated by Chiang & Knight (326), D-xylose and L-arabinose undergo the following transformations in *Penicillium chrysogenum*:

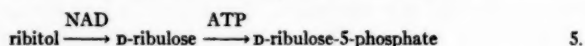


Extracts of cells grown with either D-xylose (327, 328) or L-arabinose (329, 330) as sole source of carbon contain an NADPH₂-linked aldopentose reductase for the conversion of D-xylose to xylitol and L-arabinose to L-arabitol. Attempts to demonstrate reversibility of the reactions were unsuccessful (327), although Veiga, Bacila & Horecker (331) found that the similar enzyme from *Candida albicans* catalyzes the reaction reversibly. Extracts also contained NAD-linked pentitol dehydrogenase activities for the oxidation of xylitol to D-xylulose (327) and of L-arabitol to both L-xylulose and L-ribulose (in the ratio of three to one) (329, 330). Substrate competition studies indicated that the oxidations of L-arabitol to L-xylulose and of xylitol to D-xylulose are catalyzed by one enzyme while a second dehydrogenase is responsible for the oxidation of L-arabitol to L-ribulose (326).

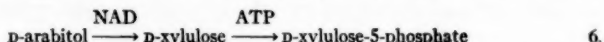
The combined action of an aldopentose reductase and a pentitol dehydrogenase has been shown to effect the transformation of D-xylose to xylitol and then to D-xylulose in a number of molds and yeasts (331, 332). L-Arabinose-grown *P. chrysogenum* also contain an induced enzyme for the NADPH₂-linked reduction of L-xylulose to xylitol. Finally, the ability of the extracts to catalyze the phosphorylation of D-xylulose, but not L-xylulose, L-ribulose, L-arabinose, D-xylose, L-arabitol, or xylitol (326), completed the enzymatic basis for the scheme of Chiang & Knight. Although the polyol-intermediate pathway has not been detected in several bacteria that have been examined (332, 333), D-arabitol formation from D-arabinose has been tentatively suggested in a proposed pathway for the conversion of D-arabinose-1-C¹⁴ to pyruvate-3-C¹⁴ in *E. coli* K-12 (334).

The ready adaptation of bacteria to growth on polyols has led to numer-

ous studies on the formation of adaptive enzymes. An interesting difference in response to pentitols has been demonstrated in two strains of *A. aerogenes*. Wood *et al.* (335) have found that *A. aerogenes*, strain PRL-R3, when grown on ribitol, forms systems for the oxidation of ribitol and for D-ribulose phosphorylation with ATP as phosphate donor. The first enzyme is a specific NAD-linked ribitol dehydrogenase that forms D-ribulose. No other substrates were found, and the kinase also was specific for D-ribulose:



Growth on D-arabitol also yielded a very specific dehydrogenase and kinase:



L-Arabitol and xylitol induced high levels of ribitol dehydrogenase, while ribitol, but not L-arabitol or xylitol, induced the formation of D-arabitol dehydrogenase.

A. aerogenes 1033 has been shown by Lin (336) to produce a D-arabitol dehydrogenase of relatively broad specificity. The enzyme is NAD-linked and converts D-arabitol to D-xylulose. Mannitol is also a substrate, but the enzyme is not induced by ribitol or by mannitol, which supports growth of the organism. The induction by D-arabitol is actually repressed by the hexitol. Anaerobic conditions favored production of high levels of the enzyme.

Ribitol is useful in studying enzyme induction in *Azotobacter agilis* (*A. vinelandii*), in which D-mannitol dehydrogenase and L-iditol dehydrogenase can be induced (337). Ribitol induces the formation of the first enzyme, for which it is not a substrate, but does not influence the formation of the second enzyme, for which it is a substrate.

For further information on pentose-polyol interconversions and other aspects of the metabolism of pentitols, a recent review may be consulted (338).

GLUCURONIC ACID PATHWAYS

An increasing number of pathways are encountered in which glucuronic acid or UDPGA is an intermediate. Since animals, plants, and microorganisms often differ considerably in this area of metabolism, they will be considered separately.

Animals.—The role of glucuronic acid in the biosynthesis of both L-ascorbic acid and of L-xylulose is accepted (339, 340, 341), but a number of uncertainties regarding enzymatic mechanisms still exist. During the past year there has been considerable activity in the investigation of these problems, especially in the glucuronate-ascorbate pathway, the earlier stages of which are also part of the glucuronate-xylulose cycle (Figure 3).

The conversion of glucose to D-glucuronate has been demonstrated in

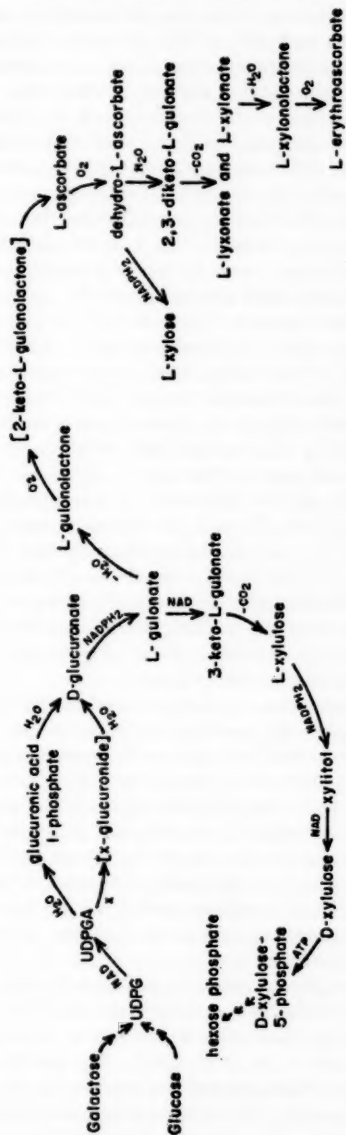


FIG. 3.—Metabolic reactions involving glucuronic and ascorbic acid.

vivo and *in vitro*. An early stage of the transformation undoubtedly involves the oxidation of UDPG to UDPGA. The successive hydrolyses of UDPGA to glucuronic acid-1-phosphate and then to glucuronate have been detected in kidney (342) and skin (343), but little evidence for the hydrolysis of glucuronic acid-1-phosphate could be found in liver (344), the organ that produces ascorbic acid. In fact, Pogell & Leloir (344) and Touster and co-workers (345, 346, 347, 347a) suggested that UDPGA may be converted to glucuronate via a reaction involving UDP-transglucuronylase (glucuronyl transferase) rather than UDPGA pyrophosphatase. Doubt as to the significance of the latter pathway arose in part from the fact that the inhibition *in vitro* of UDPGA pyrophosphatase by nucleotides increases the production of glucuronate and decreases the production of glucuronic acid-1-phosphate (344). Furthermore, barbiturates, which, *in vivo*, promote the formation of glucuronate and its metabolic products, also inhibit UDPGA pyrophosphatase *in vitro* (345, 347a). On the other hand, animals such as the cat and the Gunn rat, which are deficient in the transglucuronylase, respond poorly to agents that readily promote the production of ascorbic acid and its precursors in animals not deficient in this enzyme (346, 347). Moreover, many agents, such as aminopyrine and carcinogenic hydrocarbons, that enhance ascorbic acid production also increase the level of UDP-transglucuronylase in treated animals (346, 347, 347a, 348). There is no evidence suggesting the identity of the substrate which might yield the readily hydrolyzable glucuronide in this pathway. Further work on this problem should be facilitated by Isselbacher's (349) successful solubilization and partial purification of the transglucuronylase. It is noteworthy that the soluble enzyme is unable to catalyze the synthesis of N-glucuronides, a finding which suggests the possibility that at least two transglucuronylases occur in liver.

Although inositol is a precursor of D-glucuronate and L-gulonate, it is not converted into ascorbic acid, presumably because the acids, which are produced from inositol in the kidney, cannot penetrate the liver cells that synthesize the vitamin (350). Through the use of D-glucuronolactone-1- H^3 , Posternak *et al.* (351) have obtained the expected C-1 and C-3 labeling in urinary glucose and have been able to assess the stereochemistry of some of the enzymatic transformations involved. The tissue distribution of inositol (352), its conversion (in very small yield) to furan-2,5-dicarboxylic acid in the human being (353), and its biosynthesis from glucose in cultured mammalian cells which nevertheless require an external source of the cyclitol (354) have been reported recently.

The conversion of D-glucuronate to L-gulonate by nicotinamide-adenine dinucleotide phosphate-L-hexonate dehydrogenase has been extensively studied, most recently by Mano, Shimazono and co-workers (355, 356, 357), Ashwell *et al.* (358), and York *et al.* (359). The enzyme has such broad specificity as to suggest a more general importance in carbohydrate metabolism. The most active uronic acid is L-iduronate, which is now recognized as the uronic acid of chondroitin sulfate-B. Although York *et al.* (359), using

enzyme derived from pig kidney, found no substrate activity for D-glucuronolactone, Mano *et al.* (355, 356, 357) observed sufficient activity of the lactone with the rat liver dehydrogenase to point to the direct production of L-gulonolactone, the substrate for the ascorbic acid-forming enzyme, without the intervention of a lactonase.

L-Gulonate has two metabolic fates: (a) lactonization to L-gulonolactone followed by oxidation to L-ascorbic acid and (b) oxidation by NAD-L-gulonate dehydrogenase to yield L-xylulose. The existence of at least two lactonases in liver, together with somewhat conflicting reports on their specificity, has complicated attempts to delineate all of the transformations leading to ascorbate. Suzuki *et al.* (360) now report that lactonase I (361, 362) and aldolactonase (363, 364, 365) are identical with gluconolactonase, the enzyme hydrolyzing gluconolactone-6-phosphate to 6-phosphogluconate (366). According to Salomon & Stubbs (367), the decrease in ascorbic acid production in hypophysectomized rats can be attributed to a specific depression in the activity of aldolactonase, the level of which can be restored to near normal by the administration of somatotropin (368).

L-Gulonolactone is converted to L-ascorbic acid by gulonolactone oxidase, presumably via 2-keto-L-gulonolactone. This enzyme has continued to be studied in several laboratories (369 to 377), but it has not been well characterized or purified to any extent. It has been solubilized from rat liver microsomes by bile salts (371, 377), by rattlesnake venom (375), and by sonic oscillation (369). The enzyme requires that the hydroxyl group on position C-2 be in the levo configuration (369). Isherwood, Mapson & Chen (378) and Isherwood & Mapson (379) believe that the metabolic intermediate serving as substrate is not L-gulonolactone but an L-gulonyl-enzyme complex. The reduced ability of liver homogenates from vitamin E-deficient rats to synthesize ascorbic acid has been attributed by Caputto and his co-workers (376) to the marked sensitivity of the enzyme to lipid peroxidation. Differentiation of the dehydrogenase and oxidase activities of the enzyme has been reported by Bublitz (375). Although gulonolactone oxidase has not been detected in human liver, Baker *et al.* (380) have reported the conversion of C¹⁴-glucuronolactone into C¹⁴-ascorbic acid in man.

The further metabolism of L-ascorbic acid has been shown by Kanfer, Ashwell & Burns (381) and by Kagawa, Mano & Shimazono (382) to involve the decarboxylation, by a rat kidney enzyme, of diketo-L-gulonic acid to L-lyxonic and L-xylonic acids. The latter, via its lactone, is converted to L-erythroascorbic acid by gulonolactone oxidase (358). L-Xylose had previously been reported to be an enzymatic product of the action of a guinea pig liver supernatant fraction on dehydroascorbic acid (383). For comparative studies on the degradation of L-ascorbic acid in bacteria, the recent papers of Kamiya (384 to 387) may be consulted.

There has been increasing study of the mechanism by which many drugs and carcinogenic hydrocarbons greatly enhance the production of ascorbic acid in rats. Evans *et al.* (388) have demonstrated recently that the conver-

sion of both glucose and galactose to glucuronate is increased by barbital or chlorobutanol (Chloretone) treatment, suggesting that these drugs influence a reaction after UDPG, but before glucuronate; and Touster *et al.* (389) showed that ethionine administration blocks the enhancing effect of barbital and of 3-methylcholanthrene on ascorbate excretion. Extracts of livers from chlorobutanol-treated (345, 347a, 390, 391) and barbital-treated (345, 347a, 390) rats have increased levels of UDPG dehydrogenase, perhaps resulting from increased stability of the enzyme in these extracts (391), elevated levels of the enzyme in the tissue, or both. Animals given other ascorbate-enhancing agents, such as aminopyrine and dibenzanthracene, have elevated levels of UDP-transglucuronylase (346, 347a). Together with the observation that barbiturates inhibit UDPGA pyrophosphatase (345, 347a), these results suggest that the increased production of ascorbic acid may result from the increased activity of the dehydrogenase or of transglucuronylase and, as mentioned earlier, that the transglucuronylase pathway to ascorbate is more important than the one involving UDPGA pyrophosphatase (344, 345, 346, 347a).

That further studies of the enhancing mechanism are required is indicated by the report that lactonase II (microsomal uronolactonase) is markedly increased by several drugs (392) and that hypnotic drugs appear to inhibit aldono-lactonase (378, 379). Moreover, it is puzzling that methylcholanthrene has little effect on glucuronate excretion (393), although barbital and chlorobutanol increase both urinary glucuronate and ascorbate levels. The ascorbate-enhancing agents undoubtedly influence a fundamental aspect of protein synthesis. The activities of many microsomal enzymes involved in detoxication are increased in liver (394), and microsomes from treated animals have increased capacity to incorporate amino acids from the medium (395), including amino acids combined with soluble RNA (396).

The NAD-dependent conversion of L-gulonate to L-xylulose has been resolved by Smiley & Ashwell (397) into a two-step process involving β -keto-L-gulonic acid as intermediate. The substrate specificity of the kidney enzyme catalyzing the dehydrogenation is very broad and has been designated β -L-hydroxy acid dehydrogenase (397). Evidence for the presence of a dithiol grouping in the enzyme has been reported (397a). Among the interesting transformations promoted by the enzyme are the conversions of L-idonic acid into L-xylulose, D-gluconate into D-ribulose, and acetoacetate into L-(+)- β -hydroxybutyrate (397, 398). The decarboxylation of the β -keto aldonic acids has been attributed to a second enzyme (397).

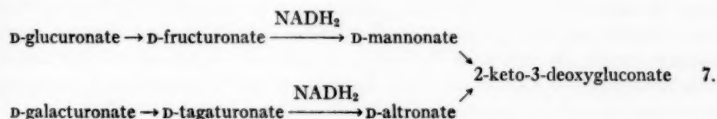
The physiological significance of the glucuronate-xylulose cycle has not been elucidated. A recent review has pointed out that the five pyridine nucleotide-linked reactions in the cycle may play a role in altering the state of oxidation of these coenzymes (341).

Plants.—Uronic acid metabolism in plants has been studied largely because of interest in ascorbic acid biosynthesis and the formation of polysaccharides. Isherwood & Mapson (378) have recently reviewed work on the

ascorbic acid biosynthesis pathway in plants and pointed out differences between the plant and animal systems. In addition, they discussed the anomalous findings of Loewus (399), who found that inversion of the carbon chain of D-glucose does not occur during its conversion into L-ascorbic acid in the strawberry fruit. In a recent note, Loewus (400) presented additional evidence that the product isolated in his study is actually L-ascorbic acid. Evidence for the conversion of L-galactono- γ -lactone into L-ascorbic acid has now been obtained in a number of higher plants (401). A study of enzymes involved in the oxidation of glucose to glucuronic acid by mung bean seedling extracts has been reported (402). Marsh (403) has also found that an enzyme system in bean leaves (*Phaseolus vulgaris*) catalyzes the reaction of UDPGA with quercitin to form quercitin β -monoglucuronide, thus demonstrating the existence in plants of an enzyme similar to mammalian UDP-transglucuronylase.

Kessler *et al.* (404) have studied the utilization of D-glucuronic acid and D-galacturonic acid by mung bean (*Phaseolus aureus*) seedlings. In addition to their conversion to pectin and hemicellulose, the uronic acids were oxidized to the corresponding aldaric acids. Both glucaric and galactaric acids are in fact natural constituents of the seedlings. Together with other reports of the presence of these substances in plants, these results suggest a possible oxidation pathway of uronic acids for energy production (404). Support for this hypothesis is also available from microbial studies (405, 406). The conversion of galacturonic acid to pectin via uridine nucleotide intermediates is indicated by its conversion into α -D-galacturonic acid-1-phosphate by extracts of young seedlings of *P. aureus* (407).

Microorganisms.—Ashwell and his co-workers (408, 409, 410) have published details of the recently elucidated pathway of metabolism in *E. coli* grown on uronic acids:



2-Keto-3-deoxygluconate is converted to the 6-phosphate derivative, which is then cleaved to pyruvate and triose phosphate. Similar enzyme systems are apparently present in *A. aerogenes* and *Serratia marcescens* which have been adapted to the uronic acids. These pathways were not detected in animal tissues or in glucose-grown cells, although glucose-grown *E. coli* cells contain a small amount of 2-keto-3-deoxy-D-gluconic kinase (410).

MISCELLANEOUS ASPECTS

Thiamine pyrophosphate in pyruvic acid catabolism.—There has been further explanation of the role of α -hydroxyethyl-2-thiamine pyrophosphate in transformations involving pyruvate. Holzer and his co-workers have

isolated the substance in experiments on the decarboxylation of pyruvate with pyruvic decarboxylase from brewer's yeast (411, 412) and on the oxidation of pyruvate by pyruvic oxidase from the mitochondria of baker's yeast (413) and pig heart muscle (414). Evidence was obtained by this group that the initial product from pyruvate (with yeast pyruvic decarboxylase) is α -lactyl-2-thiamine pyrophosphate (411). It was also shown with pyruvic decarboxylase from yeast mitochondria that the α -hydroxyethyl group of hydroxyethyl thiamine pyrophosphate is transferred to coenzyme A to yield acetyl coenzyme A (415). Carlson & Brown (416) have identified hydroxyethyl thiamine pyrophosphate as a natural constituent of microorganisms and as a product formed from thiamine pyrophosphate and either pyruvate or acetaldehyde by the action of purified wheat germ carboxylase. The conversion of the hydroxyethyl derivative to acetoin (411, 412, 416) and aceto-lactate (417) has been shown. Evidence has already been presented by Das *et al.* (418) that 2-acetyl-thiamine pyrophosphate is an intermediate in the ferricyanide-linked oxidation of pyruvate catalyzed by enzyme preparations from *E. coli*.

Tetroses and tetritols.—The activity of tetroses, tetruloses, and tetrose phosphates in enzymatic reactions, the key role of erythrose-4-phosphate in the pentose phosphate pathway and the increasingly recognized importance of polyols in metabolism have increased interest in 4-carbon sugars and their derivatives. Erythritol has now been isolated in crystalline form from the urine of fasting male human beings (419) and from bovine fetal tissues (420), where it appears to serve as a growth factor for *Brucella abortus*, an organism that grows preferentially in fetal tissue. In spite of the fact that erythritol-1-C¹⁴ is not appreciably converted to C¹⁴O₂ or glucose-C¹⁴ in rat liver slices (421), the recent discovery of the tetritol in animal tissues and fluids should encourage further work on its metabolism and physiological importance. It may be added that D-erythritol-1-phosphate and D-erythrulose-1-phosphate are interconverted by α -glycerophosphate dehydrogenase derived from rabbit muscle, the tetrulose derivative reacting at 10 per cent of the rate of dihydroxyacetone phosphate (422). Extracts of erythritol-grown *A. aerogenes* have been found to contain erythritol dehydrogenase activity (423).

In an extensive tracer study of tetrose and tetritol metabolism, Batt, Dickens & Williamson (421) have found that L-threitol and L-erythrulose are moderately well utilized by the rat, with the former substance probably first being converted into the latter. However, the labeling pattern in the glycogen synthesized was difficult to explain on the basis of known metabolic reactions. D-Erythrose-4-C¹⁴ also is metabolized, as indicated by the formation of C¹⁴O₂ and glucose-C¹⁴, with the latter having a labeling pattern (73 per cent C¹⁴ in C-6) conforming to that expected from its metabolism via a transketolase-catalyzed conversion to D-glucose-6-C¹⁴.

Nicotinamide-adenine dinucleotide phosphate-xylitol (L-xylulose) dehydrogenase (L-xylulose reductase) of guinea pig liver, previously considered to be specific for L-xylulose and xylitol (424, 425), has now been shown to

have a high order of activity towards D-threitol and D-erythrulose (426). It might be useful to investigate the metabolism of these two new substrates in mammals.

Concluding remarks.—It is regretted that outstanding advances in the chemistry of bacterial cell walls, which are frequently rich in rare carbohydrate derivatives, could not be discussed in the present review. There are several newly discovered nucleotide sugars (or related compounds) which have not been mentioned in preceding sections. Adenylyl diphosphoglyceric acid has been isolated from pig blood (427). Cleavage of this nucleotide with a pyrophosphatase yielded adenylic acid and 2,3-diphosphoglyceric acid, which is the most abundant organic phosphate in mammalian red blood cells. Uridine diphosphate dihydroxyacetone has been found in *Diplococcus pneumoniae* (428), and a guanosine diphosphate aldoheptose has been isolated from preparations of GDP-D-mannose from yeast (429). This heptose appears to be D-glycero-D-mannoheptose or its enantiomorph.

Octulose mono- and diphosphates have been isolated from normal heparinized human blood incubated with inosine and orthophosphate (430). While this is apparently the first report of the occurrence of an octulose in animal preparations, D-glycero-D-manno-octulose had just previously been isolated from avocado and sedum (431). Work on aldolase-catalyzed reactions suggests that the red cell substance may be D-glycero-D-altro-octulose (430).

An excellent monograph by Hollmann (432) on nonglycolytic pathways of glucose metabolism contains a wealth of well-illustrated theoretical and practical material.

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THE BIOCHEMISTRY OF THE WATER-SOLUBLE VITAMINS^{1,2}

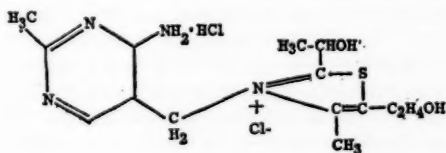
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THIAMINE

Role of hydroxyethyl thiamine in decarboxylation reactions.—Investigations have continued on intermediates involved in the decarboxylation of keto acids by carboxylase. These investigations follow the earlier findings of Breslow (1, 2), that the 2 position of thiazole of thiamine was the site of enzymatic action during decarboxylation. Subsequently, Krampitz *et al.* (3) reported on the synthesis and biological activity of hydroxyethyl thiamine. This is the compound that would be formed by decarboxylation of the addition product of pyruvate and thiamine. It was almost as active as thiamine in microbiological assays and when phosphorylated could function enzymatically in place of thiamine pyrophosphate.

The reaction between pyruvate-C¹⁴ and yeast pyruvate decarboxylase was studied by Holzer & Beaucamp (4). The reaction product, after precipitation with methanol, showed two radioactive spots upon paper chromatography which were designated "active acetaldehyde" and "active pyruvate," respectively.



Hydroxyethyl thiamine

The "active acetaldehyde" fraction was identified as hydroxyethyl thiamine pyrophosphate on the basis of the following evidence: (a) isotope experi-

¹ The survey of the literature pertaining to this review was completed in October 1961.

² The following abbreviations are used: deUMP (deoxyuridine monophosphate); FAD (flavin adenine dinucleotide); FIG (formimino glutamic acid); FMN (flavin mononucleotide); NAD (nicotinamide-adenine dinucleotide); NADH₂ (nicotinamide adenine dinucleotide phosphate); NADPH₂ (nicotinamide adenine dinucleotide phosphate, reduced form); TMP (thymidine monophosphate).

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ments showed that the 2-C but not the 1-C of pyruvate was present in the active aldehyde; (b) sulfite cleavage of the "active acetaldehyde" fraction gave a component, isolated chromatographically, in which the radioactivity was attached to the thiazole moiety; (c) treatment of the thiazole cleavage product of C^{14} -labeled "active acetaldehyde" with hydrogen iodide gave isotopically labeled propionic acid. The other isotopically labeled fraction was designated as "active pyruvate" and was characterized as α -lactyl-2-thiamine pyrophosphate on the basis of the following evidence. (a) this fraction contained C^{14} when prepared from either 1- C^{14} or 2- C^{14} pyruvate; (b) when "active pyruvate" prepared from pyruvate 1,2- C^{14} was enzymatically degraded it yielded equimolecular quantities of acetaldehyde- C^{14} and carbon dioxide- C^{14} .

Carlson & Brown (5) have identified, by paper chromatography, hydroxyethyl-thiamine derivative in extracts of various microorganisms. These extracts had been treated with phosphatase to yield the phosphate-free derivatives before chromatography and microbiological bioautographic assay. The unknown thiamine-like compound was identified as hydroxyethyl thiamine by comparison with the synthetic material by paper chromatography in several solvent systems. Hydroxyethyl thiamine accounted for ca. 60, 50, 25, and 5 per cent of the total thiamine activity of extracts of *Escherichia coli*, *Salmonella typhimurium*, baker's yeast, and *Azotobacter vinelandii*, respectively. Although hydroxyethyl thiamine is efficiently utilized by *Lactobacillus fermenti* and *L. brevis*, it was not utilized by *E. coli* mutant 26-43 as a source of thiamine. Incubation of pyruvate or acetaldehyde with wheat germ carboxylase and thiamine pyrophosphate gave a material which after dephosphorylation was chromatographically identical with hydroxyethyl thiamine. Incubation of carboxylase with α -keto-butyrate instead of pyruvate gave a new thiamine compound which was distinct from hydroxyethyl thiamine and was probably hydroxypropyl thiamine. Labeled hydroxyethyl thiamine prepared from pyruvate- C^{14} was enzymatically converted into labeled acetoin when incubated with acetaldehyde.

Effect of thiamine on transketolase activity of erythrocytes.—The role of thiamine in the transketolase system of erythrocytes has been used as a basis for evaluation of the status of thiamine nutrition. Previously Brin *et al.* (6) had shown that the oxidation of glucose through the pentose shunt by erythrocytes in the presence of methylene blue is reduced in thiamine deficiency in rats. This was measured by the accumulation of pentose in the reaction and by a reduction in the appearance of $C^{14}O_2$ from glucose-2- C^{14} . In this reaction, glucose is oxidized to glucuronate which is then decarboxylated to a pentose. Two molecules of this pentose then rearrange through a series of reactions, including a thiamine-dependent transketolase, to give a second molecule of hexose in which the 1-C is derived from the 2-C of the original glucose. The oxidation of the 1-C of the original glucose is not affected by thiamine deficiency, but formation of CO_2 from the 2-C of glucose is impaired

because it is derived only from glucose which has been formed through the transketolase reaction. It was found by these investigators that the recovery of carbon-2 of glucose as CO_2 was significantly reduced in thiamine-deficient rats, even before growth retardation was observed. Erythrocyte transketolase has been used as a criterion of thiamine deficiency for determining thiamine in foods by rat assay (7). It has the advantage of being a highly specific and also a more sensitive indicator of thiamine deficiency than growth alone. This method of assessing thiamine deficiency was extended to humans by Wolfe *et al.* (8), who found that erythrocytes of patients with Wernicke's encephalopathy exhibited reduced recovery of C^{14}O_2 from glucose-2- C^{14} . A histological condition in rats similar to Wernicke's encephalopathy in humans has also been observed in rats rendered thiamine-deficient by feeding of carp viscera (9). Although this method affords a sensitive way to detect thiamine deficiency, it has the disadvantage of requiring freshly drawn blood. An alternative enzymatic procedure has been reported by Brin *et al.* (10), in which hemolysates may be stored in the frozen state until assayed. The transketolase activity is estimated in the presence of added pentose phosphate by measuring the appearance of hexose and the disappearance of pentose. After 11 days on a thiamine-deficient diet, the growth rate of rats had not been impaired but the utilization of pentose had been reduced by 35 per cent. The specificity of this assay for thiamine deficiency was shown by stimulating transketolase activity in deficient red-cell hemolysates (as measured by pentose utilization) by addition of thiamine pyrophosphate *in vitro* to the hemolysates or *in vivo* to the rats. No stimulation was observed when thiamine pyrophosphate was added to the hemolysates of control animals receiving adequate thiamine. In contrast to the marked effect of thiamine in decreasing the transketolase activity in blood, Bagchi (11) found that thiamine deficiency in rats increased the relative activity of the shunt pathway in liver and lung but had no effect on the brain and kidney. The increase in relative activity of the shunt pathway could be the result of a reduction in the glycolytic rate rather than an absolute increase in the shunt pathway. Thiamine deficiency in the rat reduced the blood and liver glyoxalase levels. This may have been due to an accompanying decrease in tissue levels of glutathione which itself activates this enzyme [Drummond (11a)].

Distribution of thiamine phosphate esters.—Improved methods have been developed for the analysis of thiamine-phosphate esters. Rindi & De Giuseppe (12) used a Dowex 1 ion-exchange column to separate thiamine and its various phosphate esters. The relative percentages of thiamine and thiamine mono-, di-, and triphosphates were *ca.* 5, 10, 80, and 5 per cent, respectively, in rat brain, heart, liver, and kidney. When isolated rat liver is perfused with a mixture of blood and Ringers solution containing 1 to 3 mg of thiamine, both the sulfate and the phosphate esters of thiamine are formed. Barnabei & Wildemann (13) found that after one to three hours perfusion *ca.* 10 to 50 per cent of thiamine appeared as thiamine-disulfate

ester and *ca.* an equal amount as the di- and triphosphates. It is interesting that the perfusion of relatively large amounts of thiamine with an isolated liver should result in such large amounts of thiamine-sulfate ester when, under normal conditions, the phosphate esters are the predominant form of thiamine.

Microbial nutrition.—Several investigations have been reported on the nutritional requirements of microorganisms for thiamine. Weeks & Beck (14) found that *Flavobacterium aquatile* required thiamine in a medium containing salts, glucose, and trypsin casein digest. The pyrimidine and thiazole moieties of thiamine were inactive either alone or in combination. Bánhidi (15) studied the thiamine requirements of a number of heterofermentative *Lactobacilli* and their ability to utilize the phosphate esters of thiamine and its disulfides. *L. brevis* was able to utilize the homomeric disulfides of thiamine monophosphate and thiamine diphosphate and could also utilize the thiamine-R disulfide (where R was methyl, ethyl, propyl, allyl, hydroxyethyl, but not butyl or benzyl except in the presence of cysteine). Ward (16) found that a shake culture of *Pullularia pullulans* grew on a synthetic medium containing thiamine. The thiamine could be replaced by a combination of the pyrimidine and thiazole components but not by either one alone.

Thiamine absorption.—The study of thiamine and riboflavin absorption indicates that the absorption of the former is active, while that of the latter is passive. The absorption of thiamine, as measured by excretion of an oral administered dose, reaches a maximum at a dose between 2.5 and 5 mg per day (17). Riboflavin absorption is proportional to the dose administered. The absorption of thiamine administered as four doses of 2.5 mg each at 2 hr intervals is three times that of a single 10 mg dose. The administration of vitamins in various sustained-release preparations which were designed to release one-third of the dose immediately and the remainder over a 10 hr period resulted in 30 to 60 per cent increased absorption in some cases and a decreased absorption in others (17, 18).

The absorption of thiamine by the rat has been studied by Gassmann & Ketz (19), using S³⁵-thiamine. Most of the absorption occurs in the duodenum and small intestine. Thiamine was also found in the bile, mainly in the form of the mono- and diphosphate esters.

Deficiency symptoms.—Mainwood (19a) observed that reserpine had a greater effect in slowing the pulse of thiamine-deficient rats than of normals. A procedure for measuring heart rate was given which minimizes the effect of handling the animals and permits the detection of thiamine deficiency at an early stage. Dreyfus & Victor (19b) have reviewed the effect of thiamine deficiency on the central nervous system.

RIBOFLAVIN

Riboflavin analogues.—An analogue of riboflavin has been found which can apparently function as a provitamin at low concentrations and as a

reversible antagonist at high concentrations. Haley & Lambooy (20) reported that 6-chloro-7-methylflavin (6-chloro-7-methyl-9-ribityl-isoalloxazine) at low levels promoted growth in rats on riboflavin-deficient diets. Approximately maximum growth was produced by 12 μ g of riboflavin or 24 μ g of 6-chloro-7-methyl riboflavin. Slightly higher growth was produced by 50 μ g of analogue, but half the animals died within three weeks. When 500 μ g of analogue were given, the animals grew at a normal rate but all were dead in eight days. The feeding of 200 μ g of riboflavin with 500 μ g of the analogue permitted normal growth and survival. The analogue functioned as a reversible antagonist for *Lactobacillus casei* with an inhibition index of 76. These results are similar to those obtained by Lambooy (21) using diethyl-riboflavin which, at very high levels, with an inhibition index of 6 for 50 per cent reversal, functioned as an antagonist for rats. When it was administered at a level of 2 μ g/day for five days to rats on a riboflavin-deficient diet, it permitted a marked growth response for 10 days, followed by a weight loss. At the end of 28 days the weight of the treated animals was less than the controls. The authors believe that 6-chloro-7-methylriboflavin and diethyl-riboflavin function as growth promoters by

the displacement of bound riboflavin from the tissues of the deficient animals. The liberated riboflavin, which formerly had been immobilized in an animal destined to die from riboflavin deficiency, has been made available for critical metabolic requirements.

This explanation is based on the assumption that the analogue has no ability to function as riboflavin in enzyme reactions, but that it allows a riboflavin-deficient animal to use up its riboflavin reserves at a faster rate, permitting growth for a short time, after which the animal dies of acute riboflavin deficiency.

In contrast to the pro-riboflavin activity of diethylriboflavin, Lambooy & Aposhian (22) found that 6-ethylriboflavin (6-ethyl-9-ribityl-isoalloxazine) is devoid of growth-promoting activity in rats on a riboflavin-deficient diet. It functions as a low-potency antagonist for rats with an inhibition ratio of 400. It has 3 per cent of the activity of riboflavin for growth and acid production in *L. casei*. The two flavins, 6-ethyl-7-methyl- and 6-methyl-7-ethyl-riboflavin were found by Lambooy (23) to be equal to riboflavin in the nutrition of *L. casei* and ca. 40 per cent as active as riboflavin for the growth of rats. Although these two analogues could completely replace riboflavin for growth, they did not meet the nutritional requirements for reproduction (23a).

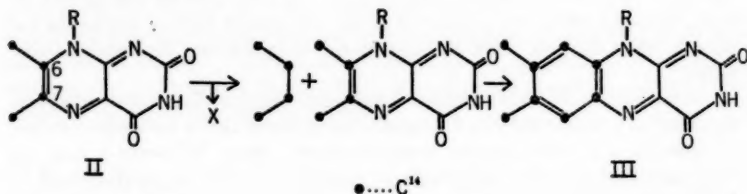
Monoamine oxidase in riboflavin deficiency.—Monoamine oxidase has been classified as a yellow enzyme (24) and a reduction in the *in vitro* monoamine-oxidase activity in liver occurs in riboflavin deficiency. Wiseman & Sourkes (25) examined the effect of riboflavin deficiency on *in vivo* oxidation of 5-hydroxytryptamine as measured by its conversion to and excretion as

5-hydroxyindole acetic acid. Dietary riboflavin deficiency, or the administration of the riboflavin antagonist, galactoflavin, did not decrease the excretion of 5-hydroxyindole acetic acid following the intragastric administration of 5-hydroxytryptamine. Riboflavin deficiency led to a 6 per cent reduction in the monoamine oxidase of the particulate fraction of liver and a 25 per cent decrease in that of the supernatant. On the basis of these observations, the authors suggest that the major metabolism of 5-hydroxytryptamine *in vivo* is other than that of the monoamine-oxidase route.

A riboflavin deficiency in the rat produced a marked reduction in the norepinephrine and epinephrine contents of the liver, but no change in the concentration of these bases in adrenals, brain, or spleen [Sourkes *et al.* (26)].

Methods of analysis.—New methods have been proposed for the analysis of riboflavin. Breyer & Biegler (27) proposed the use of alternating-current polarography to estimate riboflavin in pharmaceutical solutions and biological materials at levels of 0.1 to 1 $\mu\text{g}/\text{ml}$. Raut, Chitre & Dasai (28) developed an improved fluorometric method for determination of riboflavin in cereals. Interfering pigments are partially removed by oxidation with potassium permanganate, and reduction with zinc dust is used to reduce riboflavin to a nonfluorescing form. Riboflavin content is estimated by the difference in fluorescence before and after reduction with zinc.

Riboflavin biosynthesis.—Cell-free enzyme systems are able to convert 6,7-dimethyl-8-ribityllumazine II into riboflavin III (29). Even though the conversion of II into III involves the addition of four additional carbon atoms, no other carbon source is required besides the 6,7-dimethyl-8-ribityllumazine itself. Plaut (29) has shown by means of C^{14} -methyl-labeled 6,7-dimethyl-8-ribityllumazine that the additional four carbon atoms come from a second molecule of II. Similar conclusions have been reached by Goodwin & Horton (30) who prepared the lumazine intermediate labeled in four places with C^{14} , in the 6,7- and in the methyl groups on the 6,7-positions. The labeling of the riboflavin formed is in agreement with the reaction as shown in Equation 1, in which eight labeled carbons appear in the resulting riboflavin. The biological synthesis of riboflavin in *Eremothecium ashbyi* in a purified medium has been studied by Osman & Soliman (31). Raut & Chitre (32) observed a 1.5- to 3-fold increase in riboflavin content during the germination of cereals and pulses.



Absorption.—Chen & Yamauchi (33) studied the absorption of riboflavin, FMN, and FAD by isolated segments of surviving rat intestine. The presence of these three substances in the tissues was determined histologically by fluorescence after suitable enzymatic and extraction techniques which permitted the distinction of the three forms of riboflavin. By this procedure it was found that riboflavin and FMN were readily absorbed by the surviving rat intestine and FAD was poorly absorbed.

Dentofacial changes in riboflavin deficiency.—A riboflavin deficiency induced by either a riboflavin-deficient diet or by injection of the antagonist, lactoflavin, induced dentofacial changes in the young rats. Deuschle *et al.* (33a) noted that these animals, which might appear normal at birth, exhibited irregular development of jaws and malposition of the incisors by the third week of postnatal life.

PYRIDOXINE

Pyridoxine and renal calcium oxalate.—Pyridoxine deficiency in the cat increases urinary and kidney oxalate levels, and kidneys of deficient animals are characterized by renal scarring and by the presence of calcium oxalate crystalline material in the cortex (34, 35).

Gershoff & Andrus (36) have found a relationship between magnesium and pyridoxine in the development of calcium-oxalate lesions. A low magnesium diet (20 and 40 ppm of Mg) produced an increase in renal calcium oxalate lesions, gave a decreased citrate excretion, but did not markedly affect oxalate excretion. The addition of 400 ppm of magnesium to the diet largely prevented the appearance of oxalate calculi and also increased the urinary excretion of citric acid without affecting the oxalate excretion. The addition of dietary pyridoxine to the low magnesium diets decreased urinary oxalate, increased citrate, and prevented the appearance of urinary calculi. In these experiments, the addition of pyridoxine produced a marked (90 per cent) increase in urinary citrate excretion. The decreased citrate excretion in pyridoxine deficiency was prevented by the feeding of high levels of magnesium as well as by the addition of pyridoxine. Since it has been shown that citrate is largely responsible for increasing the solubility of calcium oxalate in urine (37), it seems possible that the increased urinary calculi in pyridoxine deficiency may be due to a decrease in urinary citrate levels as well as an increase in oxalate levels. The effect of large quantities of magnesium in reducing renal calculi may be due to the increased excretion of citrate. A comparison by Gershoff & Prien (38) of normal subjects and patients with recurring calcium oxalate kidney stones revealed that the normal subjects excreted more citric acid and less xanthurenic and pyridoxic acids than the patients with kidney stones. Although urinary oxalate was the same in both groups, the administration of pyridoxine caused a statistically significant decrease in oxalate levels in 16 out of 18 patients.

Pyridoxine-responsive anemia.—Three reports have appeared dealing with

pyridoxine-responsive anemia in humans. This syndrome resembles pyridoxine deficiency in dogs and swine (39) to the extent that there is a microcytic hyperchromic anemia, normoblastic hyperplasia of the bone marrow, an elevated serum iron, and hemosideroses of the liver, spleen, and bone marrow. A nutritional deficiency of pyridoxine induces a marked increase in xanthurenic excretion in rats, swine (39), and man (40) which is rapidly reduced when pyridoxine is fed. However, none of the patients described by either Raab, *et al.* (39), Medal *et al.* (41), or Verloop & Rademaker (42) had a severe pyridoxine deficiency as measured by xanthurenic acid excretion following a test load of tryptophan. These pyridoxine-responsive anemia patients received apparently normal diets and therefore obtained 1 to 2 mg per day of pyridoxine (43). The pyridoxine-responsive anemia responds only to large amounts of pyridoxine. Medal *et al.* (41) used 75 to 200 mg in a single dose. Raab *et al.* (39) found that 100 mg per day, administered intravenously, gave a better reticulocyte response than 25 mg per day orally. These pyridoxine supplements produce a marked reticulocyte response, a reduction in serum iron, and an increase in hemoglobin. When the pyridoxine supplementation was withdrawn, the anemia syndrome rapidly redeveloped. Although the administration of pyridoxine produces a hematological response, complete improvement does not always take place (39).

Pyridoxine and amino acid metabolism.—The role of pyridoxine in the decarboxylation of cysteine to taurine is shown by the observation that pyridoxine deficiency decreases the urinary excretion of taurine by 20 to 50 per cent (44). The taurine content of liver, spleen, and brain, however, was not significantly changed. Sauberlich (45) found that pyridoxine enhanced the availability of D-amino acids. In the presence of optimum amount of pyridoxine, D-methionine was as effective as the L-isomer, D-tryptophan was 50 per cent as effective as L-tryptophan, and D-valine was less than one-third as active as L-valine. The decarboxylation of tyrosine and tryptophan, which are pyridoxal-dependent, is inhibited by the alkaloid berberine. Kuwano & Yamauchi (46) found berberine to function as a pyridoxal phosphate antagonist in the decarboxylation of tyrosine by acetone-dried *Streptococcus faecalis* cells. Berberine inhibited decarboxylation only when preincubated with the enzyme at pH 7 to 8 prior to carrying out the decarboxylation reaction at pH 5.5 (pH optimum of enzyme). The inhibition was reversed by pyridoxal phosphate during the preincubation period. Similar results were obtained with the inhibition of pyridoxal phosphate-dependent conversion of tryptophan to indole by intact cells, dried cells, and cell-free systems of *E. coli* (47). Reversal of the inhibition by pyridoxal phosphate occurred only when the pyridoxal phosphate was added prior to or simultaneously with the inhibitor. No protection was afforded when the pyridoxal was added after preincubation of the enzyme with the berberine. Thus, there is an irreversible reaction between the berberine and the apoenzyme. Pyridoxal, even at high levels, would not replace pyridoxal phosphate in

reversing berberine inhibition of indole formation. The inhibition by berberine was most marked in cell-free systems. Gupta & Robinson (47a) noted that chicken liver urocanase which had been inactivated by carboxymethyl cellulose absorption or by hydroxylamine could be reactivated by pyridoxal phosphate.

Pyridoxal phosphate kinases and oxidases.—A study has been made by McCormick *et al.* (48) of the distribution and properties of pyridoxal phosphokinases. All the kinases phosphorylated pyridoxal, pyridoxamine, and pyridoxine, but the relative rates varied with the source. All the kinases required a divalent cation, either zinc or magnesium. Pyridoxamine phosphate oxidase and pyridoxal phosphate phosphatase were found in *E. coli* extracts [Turner & Happold (49)]. The oxidation of pyridoxamine phosphate to pyridoxal phosphate was followed by spectrophotometric measurements at 300 and 390 $m\mu$. Pyridoxamine has a strong absorption peak at 300 $m\mu$, while pyridoxal phosphate has a peak at 390 $m\mu$. The inhibition of pyridoxamine phosphate oxidase by mecoprine suggests that a flavin coenzyme is involved. The effect of a variety of inhibitors on phosphokinases was studied by McCormick & Snell (50). Condensation products of various hydroxylamines and hydrazines with pyridoxal were extremely potent inhibitors of pyridoxal kinase when either pyridoxal or pyridoxine was used as substrates. The corresponding derivatives of hydroxylamine and hydrazine with pyridoxal phosphate were noninhibitory. The free carbonyl reagents themselves were inactive as inhibitors when pyridoxine was used as substrate. Kinases from different sources varied in their sensitivity to carbonyl reagents. The kinase from brain was much more sensitive to these inhibitors than that from liver.

An enzyme which oxidizes pyridoxine phosphate to pyridoxal phosphate has been purified from rabbit liver by Wada & Snell (51). The purified enzyme also converts pyridoxamine phosphate to pyridoxal phosphate. Comparative rates of heat inactivation and pH optima indicate that the same enzyme is involved in both reactions. The enzyme is a flavoprotein which can be resolved from its prosthetic group and reactivated by riboflavin-5 phosphate. The role of flavin in pyridoxine phosphate oxidation is supported by the observation of Nakahara *et al.* (52) that a riboflavin-deficient diet produced a reduction in pyridoxal phosphate and pyridoxine-phosphate oxidase. The administration of a high-fat diet or short-chain fatty acids increased the riboflavin requirements and concomitantly reduced the activity of pyridoxine-phosphate oxidase. This effect was minimized by administration of additional riboflavin.

Pyridoxine and fatty acid metabolism.—Greenberg & Moon (53) found that the fatty acid pattern in plasma and erythrocytes of pyridoxine-deficient monkeys was the same as that of controls, thus offering no support for the theory that pyridoxine is involved in the conversion of linoleic to arachidonic acid [Witten & Holman (54)]. Similar results were obtained by Kirschman & Coniglio (55) and by Johnston *et al.* (56) in a study of the unsaturated

fatty acids in normal and pyridoxine-deficient rats. Kirschman & Coniglio (55) also found that pyridoxine deficiency did not reduce absorption of C^{14} linoleic acid. This is in agreement with the observations of Williams & Scheier (57) that the absorption of orally administered methyl arachidonate and its retention by the liver were the same in pyridoxine-deficient and supplemented rats. This demonstrated that the failure of methyl arachidonate to improve skin lesions in pyridoxine-deficient rats fed a diet containing 10 per cent cottonseed oil was not due to inability of the pyridoxine-deficient animals to retain the arachidonate.

Clinical pyridoxine deficiency.—Increased excretion of xanthurenic acid, which can be reduced by pyridoxine, has been observed in pregnancy (58). Brown *et al.* (58a) showed that the pattern of excretion of various tryptophan metabolites in pregnancy is different in many respects from that observed in simple pyridoxine deficiency. It is suggested that these differences are due to endocrine changes associated with pregnancy. One difference noted was an increased excretion of the 2-pyridone metabolite of niacin (N-methyl-2-pyridone-5-carboxamide) during pregnancy while no such increase occurred in the pyridoxine deficiency induced by deoxypyridoxine (59). It should be noted that different pyridoxine antagonists (isoniazid and deoxypyridoxine) give different patterns of excretion of tryptophan metabolites. Will *et al.* (60) studied the changes in two transaminases in blood during administration of deoxypyridoxine and subsequently of pyridoxine. The enzymes were glutamic-oxalacetate transaminase and glutamic-pyruvate transaminase. The level of the former and the percentage of enzyme saturation (as measured by adding pyridoxal phosphate *in vitro*) were decreased *ca.* 25 and 50 per cent, respectively, by deoxypyridoxine. The level of the latter was unchanged, but subsequent administration of pyridoxine raised both enzymes above their pre-depletion levels. Pyridoxine deficiency has also been observed in four patients with kwashiorkor [Theron *et al.* (61)], as measured by an increased excretion of xanthurenic acid following tryptophan feeding. The excretion was reduced following pyridoxine administration. The serum levels for glutamic-oxalacetate transaminase were normal and the patients showed no other clinical symptoms of pyridoxine deficiency. Hillman *et al.* (62) reported that the administration of 20 mg/day of pyridoxine during pregnancy reduced the development of dental caries during this period.

NICOTINIC ACID

Biological synthesis.—The synthesis of nicotinic acid in mammals and *Neurospora* from tryptophan has been well established. In bacteria, however, a different route exists. Ortega & Brown (63) studied the synthesis of nicotinic acid by resting cells of *E. coli*. Maximum synthesis occurred when glycerol, succinic acid, ribose, and adenine were added. With glycerol-1,3- C^{14} , C^{14} -incorporation was primarily in the pyridine ring. The 1,4-carbons of succinate went primarily into the carboxyl of nicotinic acid. The 2,3-carbons

of succinate appeared primarily in the pyridine ring, irrespective of the presence of glycerol. Thus, the carbon skeleton is made from a 3-carbon compound (glycerol or a metabolically related compound) and a 4-carbon dicarboxylic acid, which combine in such a way that one of the carboxyl carbons is lost and the other appears as the carboxyl carbon of nicotinic acid. The fact that ribose and adenine are required for maximal synthesis by resting cells suggests that the nucleoside or nucleotide may be the initial enzymatically formed nicotinic acid compound.

Niacin precursors in cereals.—For animals and certain microorganisms, niacin as it occurs in cereal products has a low biological availability which can be increased by alkaline hydrolysis. This inactive form of niacin has been designated "niacinogen." It has been obtained from rice, bran, wheat bran, and corn by Das & Gulha (64), as russet-colored, crystalline, rectangular plates containing 1 per cent niacin and with a molecular weight of 13,000 which corresponds to 1 mole of niacin per mole of niacinogen. Niacin is readily split off by mild alkali, indicating an alkali-labile ester linkage. Niacinogen also has a chromophoric moiety and a peptide which contains 17 different amino acids. The number of amino acid residues per molecule has been determined for niacinogen.

Niacin and cholesterol metabolism.—The mechanism of the effect of large oral doses of nicotinic acid in lowering blood cholesterol (65) has been further investigated. Altschul & Smart (66) found that 48 hours fasting caused an increase of 20 per cent in the serum cholesterol of rabbits. Administration of nicotinic acid during the fasting period reduced blood cholesterol levels 35 per cent compared to the fasted controls. Nicotinic acid (1 mg/ml) has been found by Gamble & Wright (67) to inhibit the *in vitro* incorporation of acetate-1-C¹⁴ into nonsaponifiable fraction by rat liver homogenate. Nicotinic acid had no effect on the conversion of mevalonic acid-2-C¹⁴ into the nonsaponifiable fraction, suggesting that the nicotinic acid exerts its effect between acetate and mevalonate.

Niacin metabolites.—In the rat, N-methyl-4-pyridone-5-carboxamide has been identified as the major metabolite of pyridine nucleotides (68). Chang & Johnson (69) found that, in the monkey, this same niacin metabolite, N-methyl-4-pyridone-5-carboxamide, is the principal metabolic product; ca. 70 per cent was excreted in this form after administration of 100 mg of C¹⁴-nicotinic acid. Only 10 per cent was excreted as the N-methyl-2-pyridone-5-carboxamide. In man, the 2-pyridone was the major metabolite, and only the 2-pyridone increased markedly after a 100 mg test dose of niacin. Zinc deficiency in chicks decreased the conversion of niacin into methyl-5-pyridone-5-carboxamide [Chang *et al.* (70)]. Zinc-deficient chicks contain about half as much pyridine nucleotides as did the controls and also had a reduced capacity to retain injected isotopically labeled nicotinic acid in the liver.

Methods of analysis.—Kotake *et al.* (71) have described an improved

method for the fluorometric determination of N-methylnicotinamide and niacin, in which ion-exchange chromatography is used to improve specificity.

BIOTIN

Whitaker & Umbreit (72) have found a direct relationship between the biotin content of yeast-enzyme preparations and their activity in catalyzing the exchange of ATP with inorganic phosphate. This supports the concept of Lynen *et al.* (73) that an energy-rich ADP-biotin-enzyme complex is formed as a first step in carbon dioxide fixation. The enzyme from yeast was not inhibited nor activated by *in vitro* addition of biotin. Plaut (74) studied the effect of biotin on the formation of malic enzyme by *Lactobacillus arabinosus*. This enzyme, which catalyzes carbon dioxide fixation to give malate, is present in reduced quantities in biotin-deficient turkeys (75), although no significant amounts of biotin could be found in the purified enzyme. Plaut (74) found that the formation of malic enzyme by *L. arabinosus* was the same whether the organisms were grown in the presence of biotin or a combination of "Tween" 80 and aspartate that can replace the biotin requirement. The formation of malic enzyme was increased ten- to twentyfold in the presence of malate. The incorporation of $C^{14}O_2$ by cell suspensions was much higher when the cells were grown with biotin than with "Tween" 80. If biotin is required for formation of malic enzyme, the requirements are much less than those for optimum $C^{14}O_2$ fixation. Lichstein & Waller (76) observed that glucose increased the uptake of biotin by *L. arabinosus*, indicating that an active absorption process is involved. Kosow & Lane (77) reported that rats maintained on a biotin-deficient egg white diet rapidly developed a reduced activity to fix $C^{14}O_2$ in propionyl-CoA-dependent reaction. Injection of biotin to the deficient animals caused a marked increase in propionyl carboxylase activity within 3 hr. A similar restoration of activity occurred when liver slices from deficient animals were incubated with biotin. No restoration of activity was achieved by biotin addition to cell-free systems.

The biotin content of barley, as measured by chick assay, is only one-third of that measured by microbiological assay, indicating a low availability for the chick. This availability is not increased by crude amylase enzyme action or by soaking in water [Wagstaff *et al.* (77a)].

PANTOTHENIC ACID

Aiyar & Screenivasan (78) have reported that a deficiency of pantothenic acid reduced the coenzyme Q content and succinoxidase activity of liver by *ca.* 20 per cent, the incorporation of labeled acetate into coenzyme Q by 50 per cent, and the incorporation of mevalonate by 30 per cent.

The purification and properties of pantothenic acid synthetase from *E. coli* have been described (79, 80). This enzyme catalyzes the condensation of D-pantoate with β -alanine to give pantothenic acid. Analytical methods are described for measuring the enzyme and modifications set forth which permit the estimation of pantoate and β -alanine.

CHOLINE

Analytical methods.—An improved method for choline analysis has been described by Ackerman & Salmon (81) in which preliminary extraction is replaced by hydrolysis with 25 per cent nitric acid. Choline is precipitated as the reineckate from alkaline solution. Sensitivity is further improved by photometric determination of the choline reineckate in alkaline solution at 303 m μ where the molar absorptancy is higher than the absorption peak in the visible region usually employed [Ackerman & Chou (81a)].

FOLIC ACID

Folic acid biosynthesis.—The mechanism of folic acid biosynthesis has been investigated by Brown *et al.* (82) and Jaenicke & Chan (83), using charcoal-treated cell-free extracts of *E. coli*. The suggested pathway of folic acid synthesis by *E. coli* involves the following reactions: 6-hydroxymethyl dihydropteridine + *p*-aminobenzoic acid \rightarrow dihydropteroic acid; dihydropteroic acid + glutamic acid \rightarrow dihydrofolic acid.

The mechanism involves the condensation of 6-hydroxymethyl dihydropteridine (2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine) with *p*-aminobenzoic acid in the presence of ATP and magnesium chloride. The resulting dihydropteroic acid combines with glutamic acid to give dihydrofolic acid. The evidence for the participation of 6-hydroxymethyl dihydropteridine is based on the finding that folic acid production is twice as great with the dihydropteridine as with the tetrahydropteridine (83) and that 6-hydroxymethylpteridine is relatively inactive compared to the tetrahydro form (82). Jaenicke & Chan (83) have pointed out that it is possible for 6-formyltetrahydropteridine to rearrange into 6-hydroxymethyl dihydropteridine by the migration of two hydrogens from the ring to the side chain. They also proposed that ATP activates the pteridine to yield a pyrophosphate ester with the 6-hydroxymethyl group. The double bond in the 5,6 position of the pteridine ring yields an allyl pyrophosphate which activates condensation with the amino group of *p*-aminobenzoic acid.

Jaenicke & Chan (83) observed that the only cofactors required for their charcoal-treated cell-free system were ATP and magnesium chloride. Brown *et al.* (82) reported that FAD and NADH₂ were also required under certain conditions, in addition to ATP and magnesium chloride. When the tetrahydro form of either 6-formyl- or 6-hydroxymethylpteridine was used, the addition of NADH₂ and NADP stimulated but was not essential for folate synthesis. However, when 6-formylpteridine was used, NADH₂ and FAD were essential for folate synthesis. Thus the role of NADH₂ must be largely in the reduction of 6-formylpteridine to the dihydro or tetrahydro form.

Biosynthesis of folic acid has also been obtained with a yeast enzyme using tetrahydrobiopterin (obtained from the eye of sepia mutant *Drosophila*) as the source of pteridine. Magnesium and ATP were used as cofactors (84).

This ability of biopterin, which has a 3-carbon side chain, to serve as a

pteridine precursor for folic acid is in agreement with the suggestion of Weygand *et al.* (85) that pteridines are first synthesized with a 3-carbon side chain. This suggestion is based on observations on the incorporation of C^{14} precursors into leucopterin by the pupae of the cabbage butterfly. Weygand *et al.* found that 2-C of leucopterin is derived from formate, while the 4a-C and 8a-C are derived from glycine (corresponding to 4-C and 5-C of guanine). Thus, the biogenesis of the pyrimidine ring of leucopterin corresponds to that of guanine. The carbons 6 and 7 of the pyrazine ring of leucopterin are derived from glucose or ribose, the latter being the more effective. Comparisons of the incorporation of glucose-1- C^{14} , glucose-2- C^{14} , glucose-6- C^{14} , and ribose-1- C^{14} into the 6 and 7 carbon atoms led to the conclusion that the 6- and 7-carbon atoms of leucopterin come from carbons 2 and 1 of ribose, respectively. This would occur if the 6- and 7-carbons of leucopterin were derived from the ribose of guanine nucleotide, by a process in which the 8-C of the imidazole ring of guanine was eliminated and the 2-C of ribose then condensed with the 7-N to form a pyrazine ring with a 3-carbon side chain in the 6 position. This conversion of guanine into pteridine is supported by the finding that the incorporation of both formate and glycine into carbons 2,4,5 of guanine is similar to the incorporation of the corresponding carbons into leucopterin. Such a mechanism of biogenesis of pteridines from guanine nucleotide would account for the fact that almost all of the naturally occurring pterins have an amino group on the 2 position, an hydroxyl in the 4 position, and a side chain, when it occurs, in the 6 position. Evidence supporting this hypothesis comes from the finding that in the synthesis of pteroyltriglutamate by *Corynebacterium* the 2-C, but not the 8-C of adenine is incorporated into the pteridine ring [Vieira & Shaw (86)]. Kidder & Dewey (86a) found that certain triaminohydroxypyrimidines (i.e., 2,6-diamino-4-hydroxy-5-formylaminopyrimidine) were active in sparing the folic acid requirement of *Tetrahymena pyriformis*.

5-Methyltetrahydrofolic acid (prefolic A).—A form of folic acid, designated prefolic A, was isolated by Donaldson & Keresztesy (87) from horse liver. Prefolic A had some of the spectral characteristics of tetrahydrofolic acid but possessed greater stability in acid solution and was active for *L. casei* but relatively inactive for *Streptococcus faecalis*. This prefolic A was oxidized to tetrahydrofolate by an FAD-dependent enzyme from hog livers using menadione as the electron acceptor, formaldehyde being formed during the reaction [Donaldson & Keresztesy (88, 89)]. Prefolic A was also formed from tetrahydrofolate and formaldehyde by the same enzyme in the presence of FAD using $NADH_2$ as the electron donor. Prefolic A appears to be identical with a folic acid intermediate found to be involved in the biosynthesis of methionine by Larrabee & Buchanan (90). This intermediate in the synthesis of methionine was formed from N^6,N^{10} -methylenetetrahydrofolate by the "205-2" enzyme in the presence of $NADH_2$ and did not give a test for formaldehyde with dimedon as did its precursor, N^6,N^{10} -methylenetetrahydro-

folate, but instead yielded one mole of methyl group per mole of compound. It is active for *L. casei* but inactive for *S. faecalis* and *Pediococcus cerevisiae*.

Prefolic A has been synthesized from tetrahydrofolate and formaldehyde by reduction with sodium borohydride [Keresztesy *et al.* (91) and Sakami & Ukstins (92)]. After crystallization as the barium salt, the compound was biologically active and had the same absorption spectral characteristics as prefolic A (91). It was *ca.* 50 per cent as active for *L. casei* as natural prefolic A. This is to be expected since the synthetic material would be expected to contain both diastereo isomers. Prefolic A appears to be 5-methyltetrahydrofolate. It contains a methyl group which could be on either the N⁵ or N¹⁰ position. The N¹⁰ position, however, is eliminated because prefolic A is not identical with N¹⁰-methyltetrahydrofolate which has been synthesized from N¹⁰-methylfolate (92).

Role of folic acid in methionine biosynthesis.—Additional information has been obtained concerning the biosynthesis of methionine in both bacterial and mammalian enzyme systems. Larrabee & Buchanan (90) and Hatch *et al.* (91a) have resolved several of the enzymes involved in the synthesis of methionine by *E. coli* when serine is used as the 1-carbon donor. The three enzymes involved are: (a) serine hydroxymethylase, which transfers a 1-carbon fragment from serine to form N⁵,N¹⁰-methylenetetrahydrofolate, where the 1-carbon fragment is at the formaldehyde level of oxidation; (b) "205-2" enzyme which reduces the N⁵,N¹⁰-methlene group to a methyl group; and (c) "B₁₂" enzyme which transfers the methyl group from the tetrahydrofolate derivative to methionine. The "205-2" enzyme is absent in a methionine-requiring mutant strain 205-2 of *E. coli*. Mutant strain 113-3, which requires either cobalamin or methionine for growth, contains the "205-2" enzyme and also the active "B₁₂" enzyme when grown with cobalamin, and contains the inactive "B₁₂" apoenzyme when grown with methionine. The "B₁₂" enzyme may be assayed using cell-free extracts of the 113-3 mutant grown with methionine and the "205-2" enzyme may be assayed using cell-free extracts of strain 205-2. It has also been possible to separate the two enzymes from cell-free extracts of strain 113-3 by fractionation procedures. A cell-free extract of *E. coli* 113-3 will synthesize methionine if it has been grown in the presence of cobalamin. However, if the cells have been grown in the presence of methionine, the cell-free extracts will not synthesize methionine until after the addition of either B₁₂ *in vitro*, or the addition of the "B₁₂" enzyme fraction obtained from cells grown in the presence of B₁₂. The complete enzyme system, consisting of the three partially purified enzymes require serine, homocysteine, pyridoxal phosphate, tetrahydrofolate, ATP, NADH₂, and FAD or FMN. The use of synthetic N⁵,N¹⁰-methylenetetrahydrofolate eliminates the need for serine hydroxymethylase, serine, pyridoxal phosphate, and tetrahydrofolic acid. Wilmanns *et al.* (93) studied methionine synthesis using a pig liver enzyme system and have proposed the following pathway: (a) formation of hydroxymethyltetrahydrofolate

from formaldehyde and tetrahydrofolate; (b) reaction of adenosine and homocysteine to give S-adenosylhomocysteine; (c) intra-oxidation and reduction of hydroxymethyltetrahydrofolate to give 5-methyldihydrofolate; (d) reaction of 5-methyldihydrofolate with S-adenosylhomocysteine to give S-adenosylmethionine with the release of dihydrofolate; (e) cleavage of S-adenosylmethionine to give methionine plus adenosine; (f) reduction of dihydrofolate by NADPH_2 to give tetrahydrofolate. This mechanism is supported by the observation that (a) ATP is not required when S-adenosylhomocysteine is used; (b) in the absence of homocysteine, a new folate derivative appears with different spectral properties, for which the structure of methyldihydrofolate has been proposed; (c) enzymatic reaction of this methyldihydrofolate with S-adenosylhomocysteine gives dihydrofolate—detected by its reaction with dihydrofolic reductase; (d) tritium in the pyrazine ring of hydroxymethyltetrahydrofolate appeared in the methyl group of methionine.

These results concerning the role of ATP are at variance with those of Sakami & Ukestins (92) who found ATP to be essential for the formation of methionine from acetylhomocysteine by pig liver enzyme system, and the ATP requirement could not be eliminated by using S-adenosylhomocysteine. The latter proved inhibitory. Sakami & Ukestins (92) have suggested that ATP could be involved in the conversion of the 5-N of the N^5 -methyltetrahydrofolate to a quaternary amine by the addition of an adenosyl group. This would activate and labilize the 5-methyl group in a manner similar to the activation of the methyl group in methionine by the formation of S-adenosylmethionine.

The results of Guest *et al.* (94) with cell-free extract of *E. coli* mutant strain 121/176 are similar to those of Larrabie & Buchanan (90) obtained with the similar strain 113-3. Cell-free extracts of this strain 121/176, which requires either methionine or B_{12} for growth, will, when grown with methionine, synthesize methionine *in vitro* when cobalamin and tetrahydrofolate are present in the reaction mixture. In these cell-free reactions, ATP, magnesium, and DPNH were required as cofactors. A strain of *E. coli* (PA-15), which does not require cobalamin and which was grown without cobalamin, synthesized methionine when an extract of heated cells was added. A combination of tetrahydrofolate and cobalamin replaced the extract. The addition of tetrahydrofolate to this extract inhibited methionine synthesis, which in turn could be restored by the further addition of cobalamin. Jones, Guest & Woods (95) found that tetrahydropteroyltriglutamate functions the same as the folic acid enzyme in the extract of heated cells in that it promotes methionine synthesis in absence of cobalamin. Its action is inhibited by tetrahydrofolate and the inhibition reversed by cobalamin. A folic acid derivative has been isolated from the extract, which appears identical with N^8 -formyltetrahydropteroyltriglutamic acid (96). When strain PA-15 is grown with cobalamin, tetrahydrofolate is not inhibitory. These

observations have led to the proposal by Guest & Woods (96) that two pathways for methionine synthesis exist—one involving a polyglutamate folic acid coenzyme, and the other tetrahydrofolate plus cobalamin. The functioning of these two pathways has been stated by Guest & Woods (96) in their review:

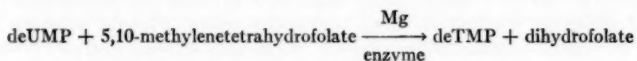
The first pathway is apparently independent of cobalamin and has a strict requirement for a polyglutamyl form of folic acid coenzyme. This pathway is inhibited by tetrahydrofolate and is blocked in cobalamin/methionine auxotrophs. The second pathway is dependent upon the metabolism of cobalamin but functions with both monoglutamate and conjugated forms of folic acid cofactor. This pathway is operative in the cobalamin/methionine auxotrophs and also in prototrophic strains which contain or can form the cobalamin-containing factor or enzyme. It is this pathway which is imposed on extracts of cobalamin-independent strains by tetrahydrofolate because it inhibits the first pathway. On this hypothesis the metabolic lesion in an auxotroph responding (for growth) to either cobalamin or methionine is in the normal pathway of methionine formation; cobalamin enables the organisms to make use of an alternative mechanism rather than, as has often been supposed, supplying the product of the blocked reaction. It is relevant that there is no record of any mutants of *E. coli* having a growth requirement for cobalamin which cannot be replaced by methionine.

Kisliuk (94a) has obtained evidence which also supports the hypothesis that organisms grown on vitamin B₁₂ are capable of synthesizing methionine by a totally different mechanism than organisms grown without vitamin B₁₂. Thus, very little vitamin B₁₂ or vitamin B₁₂ protein enzyme activity is found in extracts of organisms grown without this vitamin.

Formation of "B₁₂" enzyme.—The formation of the B₁₂ holoenzyme has been studied by Takeyama *et al.* (97) and Guest & Woods (98). Takeyama *et al.* (97) obtained the B₁₂ holoenzyme from *E. coli* strain 113-3 grown with minimum quantities of cobalamin and the apoenzyme from cells grown with methionine. The formation of holoenzyme from B₁₂ and the apoenzyme required tetrahydrofolate, FAD, ATP, and magnesium. Cobalamin anti-metabolites inhibited the formation of the holoenzyme but did not inhibit the functioning of the holoenzyme in the complete methionine-generating system. Guest & Woods (98) described the formation of cobalamin-containing enzyme [factor (X)] formed by ultrasonic extracts of *E. coli* PA-15. Extracts of acetone-dried cells of this organism, grown with cobalamin, will form methionine. They will not form methionine when the cells are grown without cobalamin, unless the cobalamin-containing enzyme is added. Free cobalamin is ineffective. Ultrasonic extracts of this same strain will form a factor (X) *in vitro* in the presence of B₁₂ which, when added to extracts of acetone-dried cells (grown without B₁₂), will permit the formation of methionine; thus, the acetone-dried cells have lost their ability to form this factor (X). The factor (X) was assayed with acetone-dried cells grown without B₁₂ under assay conditions such that neither the B₁₂ nor the enzymes used in forming

(X) would give a response. Methionine was produced only when B_{12} and the enzymes had been incubated prior to assay with an extract of acetone-dried cells. Factor (X) contained cobalamin; its activity was not lost by dialysis but was destroyed by 5 min heating at 60°C. The anilide analogue of cobalamin inhibited the formation of factor (X) but did not affect its functioning once it had been formed. Coenzyme B_{12} did not replace factor (X), although it did replace vitamin B_{12} in the formation of the factor. The cofactors required for formation of factor (X) by ultrasonic extracts included ATP, Mg^{++} , and $NADH_2$. This factor (X) may be similar to that described by Hatch *et al.* (99) and Takeyama *et al.* (97). The latter found that tetrahydrofolate and FAD were also required as cofactors in the formation of the holoenzyme.

Synthesis of thymidine.—Folic acid has been shown to be involved in the synthesis of deoxythymidylic acid from deoxyuridylic acid. Humphreys & Greenberg (100) suggested that dihydrofolate was formed during the formation of the methyl group of thymine from hydroxymethyltetrahydrofolate. Thus, tetrahydrofolate served as the carrier for the single carbon fragment and also as the source of hydrogen for the reduction of the hydroxymethyl group. Wahba & Friedken (100a) studied this reaction by measuring spectrophotometrically the conversion of tetrahydrofolate to dihydrofolate. The over-all reaction using a thymidylate synthetase from *E. coli* was as follows:



When P^{32} -labeled deUMP was used, one mole of dihydrofolate was formed per mole of P^{32} -labeled thymidylate found. The dihydrofolate was also isolated chromatographically from the reaction product and identified spectrophotometrically and enzymatically with dihydrofolate reductase. No dihydrofolate was formed when deUMP was replaced by uracil, uridine, deoxyuridine, uridine monophosphate, and deoxycytidine. The tetrahydro derivatives of aminopterin and amethopterin inhibited the conversion of tetrahydrofolate to dihydrofolate by the above reaction, but aminopterin and amethopterin themselves had little effect. 5-Fluorodeoxyuridylylate was inhibitory, but 5-fluorouracil was not. Similar results were reported by McDougall & Blakley (100b) with thymidylate synthetase from *S. faecalis* R. By measuring synthesis of thymidylate- $C^{14}H_3$ from L-serine-3- C^{14} , they found that tetrahydrofolate is a substrate in the absence of pyridine nucleotides. When $NADH_2$ is present, tetrahydrofolate or dihydrofolate is active in catalytic amounts. The addition of vitamin B_{12} *in vitro* had no effect. Aminopterin did not affect the system when substrate quantities of tetrahydrofolate were used, but did when catalytic quantities were used and the dihydrofolate reductase system was operating with DPNH to regenerate tetrahydrofolate. Similar results were obtained by Whittaker & Blakley (100c)

with calf thymus extracts. Mantsavinos & Zamenhof (100d) found that a cell-free extract of a thymine auxotroph was not able to synthesize TMP from deUMP, tetrahydrofolate, and formaldehyde, whereas a reversion mutant of this auxotroph possessed the needed enzyme system.

Formate activating enzyme.—The mechanism of action of tetrahydrofolate formylase from sheep and pigeon liver has been studied by Jaenicke & Brode (101, 101a). They suggested on the basis of kinetic and exchange studies that the first step is the formation of an enzyme-phosphate from ATP. The high-energy phosphate is transferred to tetrahydrofolic acid to give an activated enzyme-bound N¹⁰-tetrahydrofolate-phosphate. This reacts with formic acid to give N¹⁰-formyltetrahydrofolate. Attempts to make an enzymatically active phosphorylated tetrahydrofolate were unsuccessful. The results agree with those of Rabinowitz & Himes (101b) which also indicate a reaction of ATP and the enzyme. However, Rabinowitz & Himes data did not support a tetrahydrofolate phosphate intermediate. When O¹⁸ formate was incubated with ATP and tetrahydrofolate the O¹⁸ appeared in the orthophosphate, indicating a bond between these two compounds.

Folic acid reductase.—Information has been obtained by Zakrzewski & Nichol (102) to show that a single enzyme is responsible for the reduction of folate and dihydrofolate. The ratio of the rates of reduction of folate and dihydrofolate, which is 1:10, remains about the same during purification of the enzyme from chicken liver. The inhibition of the enzyme by aminopterin is almost irreversible, and the amount of this antagonist required to inhibit the enzyme is the same for both steps of folate reduction. A dihydrofolate reductase, however, has been obtained by Nath & Greenberg (103) from thymus gland, which does not react with folic acid and which utilizes NADH₂ preferentially to NADPH₂.

The binding of 4-aminofolic acid analogues by folic acid reductase was reported by Werkheiser (104). Aminopterin (4-aminopteroylglutamic acid) and amethopterin (4-amino-10-methylpteroylglutamic acid) bind themselves with folate reductase in a "stoichiometric" manner. At levels of analogue where inhibition is almost complete all the drug is enzyme-bound. The drug can be freed from the enzyme complex by dialysis in the presence of folic acid but not by dialysis in the absence of folate. The folic acid reductase of tissue measured by either the minimum amount of drug required to completely inhibit enzyme action, or the amount of drug bound in a nondialyzable form, was the same as that determined by the reduction rate of folic acid. This shows that the principal binding sites for the 4-aminofolic acid analogues are on the folic acid reductase. The development of increased resistance to aminopterin during tissue culture of Sarcoma-180 cells results in a parallel increase in their content of folic acid reductase [Hakala *et al.* (105)]. The AH and AT strain cells which had become 67- and 174-fold more resistant, respectively, contained 65 and 155 times more folic acid reductase, respectively. The kinetic characteristics, the Michaelis constant, and the turnover

number per amethopterin binding site remained the same. Bertino *et al.* (105a) found dihydrofolate reductase in leukocytes of acute leukemia and chronic myelogenous leukemia but not in normal cells or those of chronic lymphatic leukemia. Willmanns (106) reported that leukemic cells from acute leukemia and chronic myeloid leukemia have a tenfold elevated folic acid formylase activity and two- to threefold increase in hydroxymethyl folate dehydrogenase activity, compared with normal leukocytes.

Preparation of tetrahydrofolic acid.—An enzymatic procedure for preparing the *l,l*-diastereoisomer of tetrahydrofolic acid has been described, using chicken liver dihydrofolic reductase (107). Chemical reduction of tetrahydrofolate by catalytic hydrogenation introduces a second asymmetric center at carbon 6 and the product, therefore, is a *dl*-mixture of the two diastereoisomers, only one of which is biologically active. The enzymatically produced tetrahydrofolate was twice as active when assayed with the formate-activating enzyme as was the chemically prepared *dl*-*l*-tetrahydrofolate. Silverman & Noronha (108) described a new method for the preparation of *dl*-tetrahydrofolate by reducing folic acid with sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) in the presence of ascorbate.

Formiminoglutamic acid excretion in folic acid and B₁₂ deficiencies.—Although folic acid is directly involved in the metabolism of formiminoglutamic acid (FIG), an increased excretion of FIG occurs in deficiencies of both folic acid and vitamin B₁₂ in rats. In vitamin B₁₂-deficient animals the excretion of FIG can be reduced by the addition of either vitamin B₁₂ or methionine (109). Spivey Fox *et al.* (110) found that chicks on a vitamin B₁₂-deficient diet containing added *L*-histidine HCl had a high excretion of FIG. This excretion dropped sharply when methionine was fed for a 24-hour period, then rebounded to more than double its original level when the methionine supplementation was discontinued. *D,L*-Homocystine, *D,L*-homocysteine-thiolactone, cystine, choline, and betaine had no effect on FIG excretion. This is in contrast to observation with rats by Brown *et al.* (111) in which methionine, *D,L*-homocysteine, and betaine (but not choline) decreased FIG excretion when added to a B₁₂-deficient diet. The decrease in excretion of FIG following administration of methionine is paralleled by a decrease in the production of C¹⁴O₂ from *L*-histidine-2-C¹⁴. The excretion of FIG has been proposed as a criterion of folic acid deficiency in man by Broquist & Lubby (112) and Lubby *et al.* (113), with the latter (113) reporting an increased excretion of FIG following a load test with histidine occurring in patients with megaloblastic anemia, but not in 18 patients with Addisonian pernicious anemia. Zalusky & Herbert (114) found that two out of six vitamin B₁₂-deficient patients with normal folic acid serum levels had a high FIG excretion which was reduced by administration of vitamin B₁₂ but not of folic acid.

Existence of polyglutamates in animal tissue.—Silverman *et al.* (115) found that leukemic cells contained N¹⁰-formyltetrahydrofolate as the primary folic acid constituent, while the host mouse liver tissue contained mainly

prefolic A. In neither case was any evidence found for polyglutamyl derivatives. The existence of folic acid polyglutamate in blood has been postulated on the basis that the folic acid derivative in blood is more active for *L. casei* than for *S. faecalis* [Herbert, V. (116)]. However, this activity could be ascribed to prefolic A, which has the same relative activity for these two organisms.

Absorption studies with tritiated folic acid.—Folic acid plasma levels (117, 118) and plasma clearance rates based on microbiological assays have been used as a method of assessing folic acid nutritional status in man. More recently, tritiated folic acid has been used in humans to evaluate absorption and retention. Anderson *et al.* (119) found that less than 35 per cent of an oral dose of tritiated folic acid was eliminated in the feces of patients with megaloblastic anemia, while more than 50 per cent appeared in the feces of patients with idiopathic steatorrhea. Johns *et al.* (120, 121) observed that an intravenous dose of tritiated folic acid, 15 $\mu\text{g}/\text{kg}$, rapidly disappeared from the blood and 30 per cent appeared in the urine within six hours. The administration of a flushing dose of folic acid, 450 $\mu\text{g}/\text{kg}$, caused a rapid rise in plasma and urinary excretion of radioactive folic acid to give a total recovery of 90 per cent in a 24-hour period. Methotrexate (amethopterin) (7.5 mg intravenously) had only a slight flushing effect. The plasma disappearance rate for normal subjects, given tritiated folate 15 $\mu\text{g}/\text{kg}$, was more rapid in patients with pernicious anemia and nontropical sprue than in normals. Urinary excretion in six hours in nontreated cases of pernicious anemia was reduced. Five-month treatment with vitamin B₁₂ alone caused the plasma-disappearance curve to return to normal but did not bring urinary excretion completely to normal [Burgin (122)].

Clinical.—Luhby & Cooperman (123) have found folic acid therapy to be effective in five out of eight patients with thalassemia major (Cooley's anemia). Although the disease is caused by a genetic defect, the cases described were complicated by a folic acid deficiency. They had marked formiminoglutamic aciduria after histidine-loading which returned to normal after oral administration of folic acid. The serum vitamin B₁₂ levels for the two cases reported were normal (*ca.* 200 and 300 $\mu\text{g}/\text{ml}$, respectively) and the serum folic acid levels were 2.7 and 4.9 $\text{m}\mu\text{g}/\text{ml}$, respectively. The administration of 20 to 60 mg of folic acid daily caused reticulocyte peaks of 16 to 20 per cent and increases in hemoglobin of 1.5 to 5 g/100 ml. These patients had a poor ability to absorb folic acid as measured by low peak serum folic acid levels following oral administration of a test dose. By means of folic acid therapy it was possible to maintain these patients for longer periods of time without transfusions. Thalassemia is characterized by acute anemia episodes. These were, in turn, characterized by formiminoglutamic aciduria after histidine-loading and a marked response to folic acid. It should be noted that although FIG excretion indicated a folic acid deficiency there was no evidence of a megaloblastic anemia in the blood or bone marrow. Herbert & Zalusky

(124) found that many patients with alcoholic cirrhosis have a folic acid deficiency as evidenced by low serum folic acid levels and FIG excretion after a histidine load test. Izak *et al.* (124a) found low serum folic acid levels (4 $\mu\text{g}/\text{ml}$) in 35 out of 64 anemic pregnant women. Low folic acid was usually associated with low serum vitamin B₁₂ and serum iron.

VITAMIN B₁₂

Nomenclature.—A schematic representation of the various components of the vitamin B₁₂ molecule and their suggested common names are shown in Figure 1.

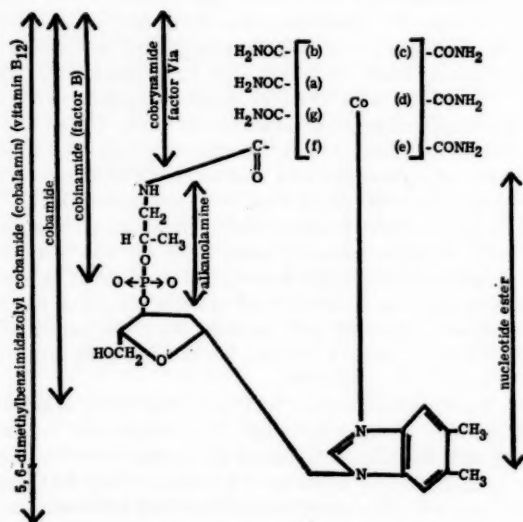


FIG. 1. Schematic structure of vitamin B₁₂ and related compounds.

Vitamin B₁₂ analogues.—Factor Via (cobrynic acid a,b,c,d,e,g-hexamide) has been isolated by Bernhauer *et al.* (125) from sewage. This vitamin B₁₂ derivative differs from cobinamide (factor B) by the absence of the aminopropanol group, and it has thus provided a valuable intermediate for the synthesis of a series of analogues based on modifications of the aminopropanol residue. The mixed anhydrides of factor Via and ethyl carbonate have been condensed with amino acids [Bernhauer & Wagner (126)], with methyl esters [Bernhauer & Dellweg (127)], and with homologues of aminopropanol [Bernhauer & Wagner (128)]. The cobinamide analogues containing serine or threonine in place of 1-amino-2-propanol were inactive for *E. coli* [Bernhauer

& Dellweg (127)], and their phosphate esters were inactive for *E. coli* and *Propionibacterium shermanii* [Bernhauer & Wagner (129)]. Since there is evidence that the aminopropanol moiety is formed by decarboxylation of threonine [Krasna *et al.* (130)], it does appear that *E. coli* is unable to effect this decarboxylation after incorporation of threonine into the cobinamide molecule [Bernhauer *et al.* (131)]. Vitamin B₁₂ analogues were prepared by condensing various cyclized nucleotides with carbobenzoxy derivatives of aminopropanol and its homologues and, after removal of the carbobenzoxy group, coupling the nucleotide ester with the mixed anhydride of factor Via and ethyl carbonate [Friedrich & Heinrich (132)].

Both the 2'- and 3'-ribose-phosphate esters are obtained by this procedure since cyclization of the nucleotide gives the 2', 3'-diester which opens up to give both the 2'- and 3'-esters. The 2'-phosphate-ribose is inactive for *E. coli* and *Ochromoneus malhamensis*. Friedrich & Heinrich (133) and Heinrich *et al.* (134) determined the biological activities of several vitamin B₁₂ analogues prepared from factor Via. These are summarized in Table I. The 2-aminoethanol derivative was 83 and 60 per cent as active as vitamin B₁₂ by the *E. coli* and *O. malhamensis* tests, respectively, and was as effective as vitamin B₁₂ in humans in the displacement from the tissues of a small dose of vitamin B₁₂ Co⁶⁰. The 2'-phosphate-ribose analogues were 2 to 5 per cent as effective as the 3'-phosphate-ribose derivatives. The 2-methyl-2-aminoethanol derivative which was inactive for *E. coli* was also inactive for *O. malhamensis* and inactive in producing a hematological response in pernicious anemia. These analogues based on modification of the aminopropanol moiety are more active antagonists than the substituted amides of vitamin B₁₂, two of which are included in Table I for comparison. It should also be noted that the substitution of a 2'-ribose-phosphate for the naturally occurring 3'-position greatly decreases the biological activity of both the provitamins and the antagonists.

Factor Via is also produced by *Propionibacterium shermanii* when grown in the absence of dimethylbenzimidazole [Bernhauer *et al.* (135)]. Under these conditions it produces large quantities of factor B and lesser quantities of a variety of nucleotide-free mono- to pentacarboxylic acids, of which the monocarboxylic acids were identified as factor Via (cobrynic acid-a,b,c,d,e,g-hexamide) and factor Vib (cobinic acid pentamide). Factor Via was converted by *P. shermanii* into vitamin B₁₂ in the presence of 5,6-dimethylbenzimidazole and into cobinamide in its absence.

Propionibacterium shermanii is capable of incorporating various bases into the nucleotide of various cobamides. The cobamides of 4,5-dimethylimidazole [Bernhauer & Muller (136)] and of 5,6-dimethoxybenzimidazole [Bernhauer *et al.* (137)] have been prepared in this way. The former compound was 50 and 8 per cent as active as vitamin B₁₂ for *E. coli* and *O. malhamensis*, respectively, while the latter cobamide functioned as a provitamin for *E. coli* and *L. leichmanii* and as an antagonist for *O. malhamensis*.

TABLE I
 BIOLOGICAL ACTIVITY OF VITAMIN B₁₂ ANALOGUES

		Phosphate Ribose Position	Growth* Promotion	Inhibition† Index
D-1-methyl-2-aminoethanol (as in vitamin B ₁₂)	$\begin{array}{c} \text{—NH H} \\ \text{H C—C—O—} \\ \text{H CH}_3 \end{array}$	3' 2'	100 2	
L-1-methyl-2-aminoethanol	$\begin{array}{c} \text{—NH CH}_3 \\ \text{H C—C—O—} \\ \text{H H} \end{array}$	3' 2'	60 2	
2-aminoethanol	$\begin{array}{c} \text{—NH H} \\ \text{H C—C—O—} \\ \text{H H} \end{array}$	3' 2'	83 5	
L-2-methyl-2-aminoethanol	$\begin{array}{c} \text{—NH H} \\ \text{H C—C—O—} \\ \text{CH}_3 \text{ H} \end{array}$	3' 2'	0.1 0.1	6:1 580:1
3-aminopropanol	$\begin{array}{c} \text{—NH H H} \\ \text{H C—C—C—O—} \\ \text{H H H} \end{array}$	3' 2'	0.01 0.01	25:1
2-methyl-2-aminopropanol	$\begin{array}{c} \text{—NH H H} \\ \text{H}_3\text{C—C—C—O—} \\ \text{CH}_3 \text{ H} \end{array}$	3' 2'	0.01 0.01	3:1 1000:1
DL-1-phenyl-2-aminoethanol	$\begin{array}{c} \text{—NH H} \\ \text{H C—C—O—} \\ \text{H C}_6\text{H}_5 \end{array}$	3' 2'	0.01 0.01	17:1
Vitamin B ₁₂ monocarboxylic methyl amide		3'		160:1
Vitamin B ₁₂ monocarboxylic anilide		3'		1500:1

* Growth promotion using vitamin B₁₂ as 100. and *E. coli* 113-3 as assay org.

† Inhibition index for 50 per cent inhibition for *E. coli* 113-3.

The site of the metabolic defect has been determined in certain vitamin B₁₂-requiring mutant strains, 466 and 959, of *Nocardia rugosa* by Di Marco *et al.* (138) and Migliacci & Rusconi (139). Strain 466 produces a substance which stimulates growth of strain 959 and which has been identified as factor Via. Strain 466 has therefore lost the ability to incorporate the aminopropanol residue. Strain 959 produces a substance which resembles the polycarboxylic acids formed by deamidation of cobinamide and is thus incapable of introducing the amide groups into cobrynic acid. Both vitamin B₁₂ intermediates which were extracted from the mycelia contained an adenine derivative and resembled cobalamin coenzyme in their absorption spectra.

There is evidence that bipterin and folic acid may be involved in the synthesis of vitamin B₁₂. Nathan *et al.* (140) reported that *Strigomonas*

oncopelti did not synthesize vitamin B₁₂ unless folic acid or 2-amino-4-hydroxy-6-trihydroxypropylpteridine was added in addition to the *p*-aminobenzoic acid. The latter met the requirements for growth but did not permit synthesis of vitamin B₁₂.

Cobamide coenzymes.—The cobamide coenzymes first isolated by Barker *et al.* (141, 142) continue to be of special interest. These coenzymes contain adenine nucleoside in addition to the base contained in the nucleotide moiety, and this adenine nucleoside is readily split off by light or cyanide. Toohey & Barker (143) reported that a major portion of the total cobamide in the hot ethanol extract of livers of chickens, rabbits, lambs, and man is in the form of coenzyme B₁₂. They further suggest that cyanocobalamin and hydroxocobalamin, which have previously been isolated from liver, are mainly artifacts resulting from chemical decomposition of the coenzyme by cyanide and by light, respectively. A sensitive ionophoretic bioautographic method has been described for detection of cobamide coenzymes. By this procedure Volcani *et al.* (144) found the coenzyme in a variety of organisms. The vitamin-requiring *E. coli* mutant 113-3 formed coenzyme B₁₂ when grown with vitamin B₁₂ and formed benzimidazolylcobamide coenzyme when grown with cobamide and benzimidazole. Five new cobamide coenzymes containing 2,6-diaminopurine, 5-methylbenzimidazole, 5-trifluoromethylbenzimidazole, 5-aminobenzimidazole, and 5-nitrobenzimidazole in the nucleotide component were obtained from *Propionibacterium arabinosum* grown in the presence of these bases [Toohey *et al.* (145)]. These coenzyme analogues were active in the glutamate- β -methyl aspartate isomerase system of *Cl. tetanomorphum*. Similar results were reported by Bernhauer *et al.* (146) for the conversion of cyanocobalamin into coenzyme B₁₂ by growing cultures or acetone-dried cells of *Propionibacterium shermanii* in a cobalt-free medium. Cobinamide coenzyme was formed in the presence of cobalt and in the absence of 5,6-dimethylbenzimidazole.

The conversion of vitamin B₁₂ to coenzyme B₁₂ has been described for cell-free extracts of *Clostridium tetanomorphum* [Weissbach *et al.* (147)] and in extracts of *Propionibacterium shermanii* [Brady & Barker (148)]. Cofactors required for this enzymatic conversion included ATP, NADH₂, glutathione, FAD, and manganese chloride. The ATP could be replaced by ADP but not by AMP. Glutathione could be replaced by mercaptoethanol, and FAD could be replaced by flavin adenine mononucleotide or riboflavin. Isotope studies with randomly labeled C¹⁴-ATP and with ATP-8-C¹⁴ showed that both the adenine and the ribose were incorporated into the nucleoside fragment of coenzyme B₁₂ [Peterkofsky *et al.* (149)].

A study by Ladd *et al.* (150) of the spectra of the benzimidazole- and adenine-containing cobamide coenzymes in neutral and acid solutions suggested that the spectral differences between the benzimidazole and adenine-containing coenzymes were due to differences in the strength of the bond between the base and the cobalt. The interpretation was drawn that the

binding of the benzimidazole moiety to cobalt in the cobamide coenzymes is not as strong as in the corresponding vitamins.

The cobalt in coenzyme B₁₂ appears to be in the divalent state, while in vitamin B₁₂ it is trivalent. This is based on the observation of Bernhauer *et al.* (151) that cyanocobalamin with a trivalent cobalt is diamagnetic, while coenzyme B₁₂ is paramagnetic, and on the similarity between reduced vitamin B₁₂ and coenzyme B₁₂. By the action of light, orange-colored coenzyme B₁₂ is readily split to give the red-colored hydroxocobalamin. When this cleavage is carried out anaerobically, a yellow-brown-colored reduced derivative is formed and adenine nucleoside is liberated. This is converted into a bright red hydroxocobalamin by atmospheric oxygen. Berhauer *et al.* (152) have suggested that the similarity in absorption spectra of coenzyme B₁₂ and reduced hydroxocobalamin indicates the presence of a divalent cobalt in the coenzyme. Brady & Barker (148) have proposed that the role of NADH₂ in the formation of the coenzyme B₁₂ is in the reduction of cobalt to the divalent state.

The sugar in the adenine nucleoside has been identified as ribose by Lenhart & Hodgkin (153), on the basis of crystallographic analysis. Barker (154) has suggested that an unidentified C-5 sugar (corrinose) is obtained by acid hydrolysis of the nucleoside obtained, in turn, by photolysis of vitamin B₁₂ coenzyme. It appears that this unsaturated sugar is formed by cleavage of the ribose-cobalt bond found during photolysis.

Vitamin B₁₂ in conversion of propanediol to propionaldehyde.—Brownstein & Abeles (157) and Abeles & Lee (158) reported the requirement of cobamide coenzymes in the conversion of 1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde by cell-free extracts of *Aerobacter aerogenes*. Adenylcobamide, benzimidazolylcobamide, and dimethylbenzimidazolylcobamide coenzymes were equally active, while cyanocobalamin itself was inactive. Brownstein & Abeles (157) also showed that in the conversion of 1,2-propanediol to propionaldehyde the hydrogen, which is transferred during the course of the reaction from C-1 to the C-2 position of propanediol, does not exchange with D₂O solvent. This eliminates the possibility of enol formation followed by ketonization as a mechanism for this reaction because this would permit exchange of hydrogen with deuterium in the water. The failure of deuterium from the solvent to be incorporated into the propionaldehyde indicates the migration of a hydride ion from C-1 of propanediol to C-2 with the concomitant displacement of the hydroxyl group. This suggests that vitamin B₁₂ may serve as a hydrogen carrier in this reaction.

Vitamin B₁₂ in propionic acid metabolism.—Vitamin B₁₂ as the coenzyme is involved in the isomerization of methylmalonyl-CoA to succinyl-CoA [Eggerer *et al.* (159); Gurnani *et al.* (160)]. Arnstein & White (161) have observed that vitamin B₁₂ increased the oxidation of propionate by resting cells of *O. malhamensis* grown on limiting amounts of this vitamin. Cobinamide and various adenyl cobamides were inactive while several substituted

benzimidazolyl cobamides were active. The anilide analogue of vitamin B₁₂ functioned as an inhibitor of both growth and propionate oxidation, while the methylamide vitamin B₁₂ analogue functioned as antagonist for growth, but as a provitamin in the oxidation of propionate. Coupled with the fact that hydroxybenzimidazolyl cobamide is active in promoting growth but slightly active in increasing propionate oxidation, this indicates that vitamin B₁₂ controls growth of *O. malhamensis* by some reaction other than isomerization of methylmalonyl-CoA to succinyl-CoA.

Nucleic acid synthesis.—The role of vitamin B₁₂ in nucleic acid synthesis appears to be at least twofold: (a) in the reduction of ribosyl to deoxyribosyl, and (b) in the formation of the methyl group of thymine. This involvement of vitamin B₁₂ in nucleic acid synthesis has been reviewed by Schweigert (162). Dinning *et al.* (163) have suggested that vitamin B₁₂ functions in the conversion of formate to thymine methyl but not in the conversion of formaldehyde to thymine methyl. An alternate role of vitamin B₁₂ in the synthesis of thymine has been suggested by Seifter & Henson (164) and also by Woolley (165). This hypothesis arose as a result of the demonstration by Barker *et al.* (166) that the formation of β -methylaspartic acid, from glutamic acid was coenzyme B₁₂-dependent. β -Methylaspartic acid could, through a series of enzymatic reactions similar to the biosynthesis of uracil, eventually yield thymine. Such a pathway of thymine biosynthesis would partially explain the known requirement of vitamin B₁₂ in nucleic acid and biosynthesis. Evidence of such a pathway is, as yet, quite limited. Isenberg, Seifter & Berkman (167) observed that β -methylaspartic acid could partly replace vitamin B₁₂ for growth of *Poteriochromonas stipitata* and could, like thymine, prevent inhibition of growth by urethan. Subsequently, Seifter *et al.* (168) reported that the cell yields of *Poteriochromonas*, *Ochromonas*, and two *Euglena* species were markedly increased by β -methylaspartate in the absence of exogenous vitamin B₁₂. With *Lactobacillus leichmannii* no effect of the amino acid could be detected. These same workers also obtained reticulocyte response in pernicious anemia patients to one gram of β -methylaspartic acid per day. With rats fed a diet containing 0.1 per cent iodinated casein, but devoid of vitamin B₁₂, no effect due to the amino acid was observed. Woolley & Koehelick (169) reported that *Escherichia coli* grown in the presence of tritiated β -methylaspartic acid incorporated the isotope into thymine. Johnson & Webb (170) using glutamic-3,4-C¹⁴ acid and β -C¹⁴H₃-aspartic acid showed that the proposed pathway is not operative in partially hepatectomized rats.

Manson (171) has found that conversion of the ribose of C¹⁴-labeled guanosine to deoxyribosides by *Lactobacillus leichmannii* is vitamin B₁₂-dependent. Similar results have been observed in cell-free extracts of mouse lymphoblast grown in tissue culture [Manson (172)], which converted the ribose of guanosine-C¹⁴-nucleotide into deoxyribosides in the presence of NADPH₂. Extracts inactivated by charcoal treatment could be reactivated

by addition of vitamin B₁₂ or coenzyme-B₁₂. It is interesting that vitamin B₁₂ in this system was as active as the coenzyme in the conversion of ribosyl to deoxyribosyl.

Effect of vitamin B₁₂ on carbohydrate and fat metabolism.—Considerable evidence has been presented in the literature to suggest involvement of vitamin B₁₂ in carbohydrate and lipid metabolism (173 to 177). Chang *et al.* (178) and Chow (179) found that a deficiency of vitamin B₁₂ increased the ratio of NAD/NADH₂ in rat liver twofold, but had no effect on the total NAD+NADH₂ content. The blood pyruvate and lactate levels were also increased by 50 and 100 per cent, respectively, in the deficient animals. Biswas & Johnson (180) found a decreased incorporation of radioactive glucose, serine, and alanine into the glycogen of vitamin B₁₂-deficient rats. No effect was observed on the incorporation of these precursors into liver lipid, but the incorporation of serine into glycerol was markedly depressed.

O'Dell *et al.* (181) observed no effect of vitamin B₁₂-deficiency in rats on the rate of anaerobic glycolysis in liver and brain tissue. A 10 per cent decrease occurred in liver nonprotein-SH compounds but no change in nonprotein-S-S compounds was found in vitamin B₁₂ deficient weanling rats. In the livers of newborn vitamin B₁₂-deficient rats there was a decrease in nonprotein-SH but a corresponding increase in nonprotein-S-S showing that vitamin B₁₂ is concerned in maintaining these sulfhydryl compounds in the reduced state.

The lipid material of vitamin B₁₂-deficient chick embryos contained a markedly higher triglyceride content and lower cholesterol ester content than embryos from supplemented hens [Moore & Doran (182)]. No change in the phospholipid composition occurred. This change in lipid composition parallels the increase in neutral fat and the decrease in cholesterol ester which occurs in plasma lipids of pernicious anemia patients (183).

Intestinal and rumen synthesis of vitamin B₁₂.—The role of coprophagy by the rat in the utilization of vitamin B₁₂ formed in the lower part of the intestine is shown by the studies of Morgan *et al.* (184) on the vitamin B₁₂ levels of stomach contents of rats. The stomachs of those rats practicing coprophagy contained 15 $\mu\text{g/gm}$ of stomach contents while for those in which coprophagy had been prevented the content was 0.8 $\mu\text{g/gm}$. The feeding to sheep of a cobaltic oxide bullet, which remains in the rumen, increased the vitamin B₁₂ content of ewe milk by three- to tenfold [O'Halloran & Skerman (185)].

Vitamin B₁₂ requirement and thyroid function.—The feeding of thyroid or iodinated casein as a source of thyroxine-like activity has been used as a method of producing vitamin B₁₂ deficiency in rats and chicks. It is difficult to reconcile this with the recent observation of Okuda & Chow (186) that thyroidectomy decreased absorption of vitamin B₁₂, and the addition of thyroid to normal animals increased absorption and increased vitamin B₁₂ levels in the liver. The involvement of the thyroid in vitamin B₁₂ absorption

is supported by the observation that low vitamin B₁₂ serum levels have been found in cretins [Hellegers *et al.* (187)]. Chow *et al.* (188) have also reported that the inability of thyroidectomized animals to become pregnant while maintained on a stock diet was partially overcome by administration of additional vitamin B₁₂, and almost completely corrected by a combination of vitamin B₁₂ plus desiccated thyroid. Thus, there is an apparent increased requirement for vitamin B₁₂ for reproduction in thyroidectomized animals, which is in contrast to the increased vitamin B₁₂ requirement for growth produced by feeding thyroid to normal animals.

Absorption of vitamin B₁₂.—Holdsworth & Coates (189) have confirmed with rats the finding of Booth & Mollin (190) in humans that the ileum is the principal site of vitamin B₁₂ absorption. A variety of nutritional deficiencies affect vitamin B₁₂ absorption. Iron deficiency in rats causes a decrease in intestinal absorption of vitamin B₁₂ Co⁹⁰ and a 40 per cent decrease in tissue uptake of the vitamin [Yeh *et al.* (191)]. Pyridoxine deficiency decreased vitamin B₁₂ absorption, while a deficiency of thiamine, riboflavin, niacin, or pantothenic acid had no effect. A deficiency of ascorbic acid in the guinea pig increased the hepatic uptake of vitamin B₁₂, but had no effect on the uptake by other organs [Hsu *et al.* (192)]. The absorption of labeled vitamin B₁₂, as measured by urinary excretion tests, was found to be enhanced twofold in humans when it was given with food and, as measured by tissue uptake, twofold in rats [Seigel *et al.* (193)]. Deller *et al.* (194) found a similar increase in gastrectomized patients but none in pernicious anemia patients. The secretion of bile may also affect vitamin B₁₂ absorption. Johnson *et al.* (194a) found that absorption of vitamin B₁₂ by the rat was more rapid when the intestine was ligated below the bile duct, that the administration of bile inhibited vitamin B₁₂ absorption, and that this inhibition could be overcome by administration of rat gastric juice.

Properties of intrinsic factor.—Porcine intrinsic factor concentrates have been prepared which are active at a level of 40 μ g combined with 1 μ g of vitamin B₁₂ [Bromer & Davisson (195)]. This was prepared from pancreatin-treated hog pyloric mucosa, by means of chromatography on ion-exchange resin, then on calcium phosphate gel, and by ultracentrifugation with an excess of vitamin B₁₂. The final material appeared homogeneous, sedimented with an S_{20} of 4.4×10^{-13} , and contained 6.8 per cent reducing sugars and 25 μ g vitamin B₁₂ per mg. This would correspond to a molecular weight of 53,000 based on 1 mole of vitamin B₁₂ per mole of complex. Faillard *et al.* (196) found that neuraminidase from *Vibrio cholera* culture filtrate destroyed 80 to 90 per cent of intrinsic factor activity of the mucoid fraction obtained from human or porcine gastric mucosa and concurrently liberated 65 to 75 per cent of the mucoid-bound neuraminic acid. An *in vitro* test for pernicious anemia has been proposed by Gulberg (196a) and by Glass *et al.* (197) based on the electrophoresis of gastric juice previously incubated with vitamin B₁₂. The vitamin B₁₂ bound in the "primary binder" electrophoretic fraction was

1.1 $\mu\text{g}/\text{ml}$ of gastric juice in pernicious anemia patients, and 14 $\mu\text{g}/\text{ml}$ in normals.

Although intrinsic factor is concerned with the transfer of vitamin B₁₂ across the intestinal wall it also promotes the binding of vitamin B₁₂ to liver slices (198) and to liver homogenates by a calcium-dependent reaction. This has raised the question as to whether intrinsic factor has an extra gastrointestinal function. In support of this view is the observation of Herbert (199) that the introduction of hog intrinsic factor into the blood stream of the rat increased liver uptake of vitamin B₁₂. Similarly, Toporek (200) found that intrinsic factor increased the uptake of the vitamin B₁₂ by the isolated perfused liver, and that the intrinsic factor could be detected in the bile. It was noted by Castro *et al.* (201) that the absorption of vitamin B₁₂ by liver homogenate in the presence of intrinsic factor can be blocked by purified blood group A or B substances, by human gastric juice from A, B, or O donors, and by *Pneumococcus* polysaccharide type XIV. These inhibitors are most effective when preincubated with the liver homogenate, which suggests that the inhibitors act by attaching themselves to the liver receptors, preventing the further uptake of intrinsic factor. These inhibitors could be removed by incubation in sodium chloride solution.

Vitamin B₁₂-releasing factors.—There is evidence to indicate that a releasing factor functions to liberate the vitamin B₁₂ from the intrinsic factor complex. Thus Herbert (202) has found that the addition of rat intestine extract will liberate (render dialyzable) vitamin B₁₂ bound to rat intrinsic factor. There is a species specificity of vitamin B₁₂ release in that human intestinal extract will not liberate vitamin B₁₂ from the rat intrinsic factor vitamin B₁₂ complex and rat intestinal extract will not liberate the vitamin from the human intrinsic factor vitamin B₁₂ complex. It was suggested that the species specificity of intrinsic factor may not only involve the reduced binding of the heterologous B₁₂-intrinsic factor complex to the receptors on the host intestine, but also the failure of the vitamin to be released from this complex by the species specific host intestinal releasing factor. There is also some clinical evidence that malabsorption of vitamin B₁₂ can be due to a deficiency of the releasing factor in the intestine, as well as due to a deficiency of the intrinsic factor in the stomach. Colle *et al.* (203) described a child with macrocytic anemia and impaired vitamin B₁₂ absorption but who had a normal gastric mucosa and intrinsic factor in the gastric juice. Administration of intestinal juice from normal patients promoted absorption. The patient did not have a generalized malabsorption, and it was postulated that the absorption defect resided in a deficiency of the releasing factor. Nyberg (204) found this releasing factor in a variety of human tissues, such as kidney, liver, and thyroid, as well as in the intestine. These organ extracts would also liberate bound vitamin B₁₂ from human serum or plasma. Further evidence for the release of the vitamin B₁₂ before absorption lies in the observation of Reizenstein *et al.* (205) that labeled vitamin B₁₂ is not ab-

sorbed through the lymphatic system. Since large molecules, including native proteins, are generally absorbed through the lymph, it does not appear that the vitamin B₁₂-intrinsic factor complex is adsorbed directly (205).

Resistance to intrinsic factor.—Although crude gastric pylorus has been used successfully for two decades in treatment of pernicious anemia, the more recent use of hog intrinsic factor concentrates prepared from hog pyloric mucosa has resulted in hematological relapse in some patients after prolonged use. The cause of the failure of the newer oral preparations in these patients refractory to hog mucosal intrinsic factor appears to be a block in intestinal absorption of vitamin B₁₂ that does not occur when human or rat intrinsic factor is used [Schwartz *et al.* (206)].

It has been suggested that this resistance to hog mucosal preparations was the result of an immunological reaction, since the sera of patients refractory to hog mucosal intrinsic factor would, when mixed with hog pyloric mucosa, inactivate the intrinsic factor and prevent the absorption of vitamin B₁₂ as measured by the Schilling urinary excretion test in another pernicious anemia patient [Schwartz (207)]. In a detailed study of this phenomenon, Schwartz (208) found that the inhibitory factor was absent in sera of 39 normal patients, and that it was present in approximately half of the patients who had received either crude stomach preparations or hog pyloric mucosa and in *ca.* 25 per cent of untreated pernicious anemia cases. The antibody resides in the globulin fraction of the blood and does not affect the binding of intrinsic factor to vitamin B₁₂ and therefore only involves the binding of the intrinsic factor with the intestinal receptors. This antibody also inactivates human gastric juice and rat gastric juice *in vitro*. This is in contrast with the oral *in vivo* effectiveness of both human and rat gastric juice in overcoming blockage in refractory patients. The absence of inhibitory factor in the sera of normal patients and its presence in 25 per cent of untreated pernicious anemia patients has led to the suggestion by Schwartz (208) that pernicious anemia may be an autoimmune disease. Lowenstein *et al.* (209) found antibodies in the sera of five patients refractory to hog pyloric mucosa. These antibodies were not absorbed by nongastric hog protein nor by human gastric juice but only by hog preparations with intrinsic factor activity. The antibodies from rabbits immunized with hog intrinsic factor combined with the vitamin B₁₂-binding portion of hog pyloric extract and altered its electrophoretic mobility. This evidence points toward a genuine antibody response to the intrinsic factor component of the hog pyloric extract.

Vitamin B₁₂ blood levels and excretion rates.—The injection of hydroxocobalamin produces a higher and more prolonged elevation of serum-vitamin B₁₂ levels in dogs than cyanocobalamin [Skeggs *et al.* (210)]. This may have been due to the observed higher binding capacity of dog serum for hydroxocobalamin than for cyanocobalamin. Schilling *et al.* (211) also found that human liver protein and serum had a higher binding capacity for hydroxocobalamin than for cyanocobalamin. Both forms were equally bound by

gastric juice and were absorbed equally well when mixed with gastric juice. Similar observations have been made by Heinrich & Garbe (212), Samson *et al.* (213), and Glass *et al.* (214). Doses of 500 to 1000 μg of hydroxocobalamin were two to three times better retained than similar doses of cyanocobalamin. Lee & Glass (214a) reported that the hepatic uptake of orally administered coenzyme B₁₂ Co⁵⁸ was not as rapid as cyanocobalamin. Spivey Fox *et al.* (214b), however, found that coenzyme B₁₂ was equally active with cyanocobalamin in promoting growth of chicks.

A vitamin B₁₂ peptide complex from liver was found to be absorbed twice as efficiently as vitamin B₁₂ in gastrectomized patients, but was absorbed to the same extent as vitamin B₁₂ in normal patients [Milhaud (215)]. Hedbom (215a) found that a vitamin B₁₂ peptide complex from bovine liver, which appeared electrophoretically homogeneous and which had a molecular weight of about 10,000, contained 13 amino acids and a total of 80 amino acid residues. Watkin (216) found that no urinary excretion of vitamin B₁₂ occurred at plasma levels up to 1.0 $\text{m}\mu\text{g}/\text{ml}$. This coincided with the vitamin B₁₂-binding capacity of the plasma of 1.0 $\text{m}\mu\text{g}/\text{ml}$. When this plasma level was exceeded, urinary excretion occurred which was proportional to the amount in the plasma at levels of 12 to 26 $\mu\text{g}/\text{ml}$. The vitamin B₁₂ clearance rate was the same as the glomerular filtration rate.

Biological half life and daily requirement.—Heinrich (217) has measured the biological half life and exchange rate of vitamin B₁₂ Co⁶⁰ by periodic radioactivity measurements in a whole-body counter and used this to determine the minimum daily requirements of the vitamin. The biological half-life after 100 days was calculated to be 500 days and the exchange rate (based on a total-body pool of 5000 μg vitamin B₁₂) was 7.0 $\mu\text{g}/\text{day}$. After 500 days, the calculated half-life was 1300 days and the exchange rate 2.7 $\mu\text{g}/\text{day}$. The loss in vitamin B₁₂ from the body corresponded to that amount which was lost daily through the feces. This exchange rate represents the loss of vitamin B₁₂ from the body and corresponds to a minimum daily requirement for vitamin B₁₂ of 3 to 6 $\mu\text{g}/\text{day}$.

Following the injection of Co⁶⁰-vitamin B₁₂, two analogues of vitamin B₁₂ were found in the bile which accounted for 25 per cent of the total radioactivity, the remainder being accounted for as vitamin B₁₂ [Stokes (218)]. No ionic Co⁶⁰ was formed either in the bile (218) or in the liver, following injection of cyanocobalamin Co⁶⁰ into dogs [Rosenblum *et al.* (219)].

Clinical.—The use of radio-vitamin B₁₂ as a clinical diagnostic tool has been reviewed by Heinrich (220, 221). Reisner (222) has presented evidence for an induced hemolytic factor in pernicious anemia. This is based on the observation that the half-life of normal chromium-labeled red cells is shortened when injected into a pernicious anemia patient and that this is increased after the patient has been given vitamin B₁₂.

Vitamin B₁₂ deficiency has been associated with fish tapeworm (*Diphyllobothrium latum*) infestation. The symptoms of the anemia induced by this

parasite resemble those of pernicious anemia. When the tapeworm inhabits the upper part of the intestine, anemia occurs more frequently than when the tapeworm is lower down [von Bonsdorff (223)], and it has been suggested that the vitamin B₁₂ deficiency is caused by uptake of the vitamin by the parasite. Nyberg *et al.* (224), in a survey of 1300 patients in Finland, found that 27 per cent were worm carriers and that 2 per cent of the carriers had megaloblastic anemia. The average vitamin B₁₂ serum levels of the noncarrier and carrier groups were 0.27 and 0.12 $\mu\text{g/ml}$, respectively. An unidentified, folic acid-like compound with *P. cerevisiae* activity was found in the urine of an untreated juvenile pernicious anemia patient by Clement *et al.* (225). This component could not be detected after treatment with vitamin B₁₂. This suggests a role of vitamin B₁₂ in the interconversion of folic acid derivatives. An elevated unsaturated plasma vitamin B₁₂-binding capacity has been observed in chronic myelocytic anemia [Weinstein *et al.* (226)]. Weinstein & Watkin (227) have found that the high vitamin B₁₂ serum level of patients with myelocytic leukemia—a level 20 to 30 times normal—is due to a different tissue distribution and not to an increased absorption of orally-administered vitamin B₁₂.

Assay of vitamin B₁₂.—A method using "saturation analysis" for the assay of vitamin B₁₂ in plasma has been proposed by Rothenberg (228) and by Barakat & Ekins (229). This procedure is based on the principle that the ratio of bound to free vitamin B₁₂ in equilibrium with a given quantity of intrinsic factor (or other vitamin B₁₂-binding agent) is a function of the total amount of vitamin B₁₂ present. In practice, the vitamin B₁₂ from a sample of deproteinized plasma (containing a trace of isotopically labeled vitamin B₁₂) is added to a given quantity of intrinsic factor, the protein-bound B₁₂ is precipitated, and the amounts of free vitamin B₁₂ in the filtrate and bound vitamin B₁₂ in the precipitate are determined by counting. The ratio of bound to free vitamin B₁₂ is then compared with the ratio observed when different levels of vitamin B₁₂ are added to the same quantity of intrinsic factor.

ASCORBIC ACID

Deficiency symptoms and metabolic changes.—The excretion of *p*-hydroxyphenylpyruvic acid by guinea pigs, following ingestion of large amounts of tyrosine, is increased in ascorbic acid deficiency and the amounts of ascorbic acid required to depress the excretion to the maximum extent is larger than that required for prevention of other scorbutic lesions. Goswami & Knox (230) found that the increase in excretion was due to a tyrosine-induced increase in tyrosine-ketoglutarate transaminase and a decrease in the phenylpyruvic oxidase. The decrease in oxidase was due to a reversible inactivation which could be relieved by ascorbic acid and indophenol dyes such as 2,6-dichlorophenol. Thus, effectiveness of ascorbic acid in reducing the excretion seems to reside in a function different from its scorbutic effect.

Pigmentation of the gingiva, which can be reduced by ascorbic acid feeding, has been observed in scorbutic children. A study of the effect of ascorbic acid on the oxidation of DOPA has led to the suggestion by Matsuzawa (231, 232) that this pigmentation is due to an inhibitory effect of ascorbic acid on the DOPA-DOPA chrome oxidation reaction to give melanin.

Biosynthesis of ascorbic acid.—Chatterjee & Kar (233) reported that deficiencies of folic acid, riboflavin, pyridoxine, and pantothenic acid reduced by 60 to 90 per cent the synthesis of ascorbic acid by rat liver microsomes. Addition of these in vitamins *in vitro* did not activate the deficient systems, and addition of antivitamins *in vitro* did not depress synthesis by normal tissue. Srinivasan *et al.* (234) reported that increasing dietary protein levels increased liver, adrenal, and blood ascorbic acid levels and increased urinary ascorbic acid excretion. A twofold increase in urinary ascorbic acid levels and reduction in hepatic ascorbic acid destruction rate have been observed in alloxan diabetes by Kanda & Sakamoto (235). Administration of insulin restored both the urinary excretion level and the hepatic ascorbic destruction rate to normal. Suzuki *et al.* (236) compared a variety of analogues of L-gulonolactone for their activity as precursors for synthesis of ascorbic acid by rat liver microsomes. They found that L-galtonolactone was 67 per cent as effective as L-gulonolactone, and other lactones were less effective.

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FAT-SOLUBLE VITAMINS^{1,2}

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Compared with the state of knowledge twenty years ago (1), much more is now known about the chemistry of fat-soluble vitamins, but despite intense world-wide activity many fundamental biochemical questions have not been answered.

From among the very numerous papers which came under scrutiny we decided to select those which aimed at elucidating a mode of action or some related biochemical topic. This has meant, to our regret, making no mention of many valuable papers dealing with more narrowly chemical aspects, with analytical procedures, or with nutritional and physiological matters not directly bearing on the main theme, i.e. function, displayed through explicable, or partially explicable, molecular processes.

Definitive rules for the nomenclature of vitamins have been published by the International Union of Pure and Applied Chemistry (2). According to these rules, vitamin A₁, vitamin A₂, retinene, and vitamin A acid will be known as retinol, 3-dehydroretinol, retinal, and retinoic acid, respectively. Vitamins D₂ and D₃ become ergocalciferol and cholecalciferol. The tocopherol usage should be adopted for vitamin E-active substances. For the vitamin K group, it is suggested that phyloquinone be used for K₁ and farnoquinone for K₂.

In a transitional period, problems of nomenclature are specially difficult for a review article. We have decided to use older terms when wide acceptance of new ones appears to be uncertain (vitamin A) or has been overtaken by new developments (vitamin K). On the other hand, we have consistently employed the new nomenclature when it is unambiguous and seems to be increasingly in use (vitamins D and E).

VITAMIN A

The papers delivered at a symposium held in May 1960 in honour of Professor P. Karrer have been published. They cover much of the vitamin A field and include the synthesis and labelling of vitamin A and related compounds (3); their physico-chemical (4) and biological assay (5); the conversion of β -carotene to vitamin A (6); the absorption, transport, and storage of vitamin A (7); its metabolic transformations (8); the visual functions of the

¹ The survey of the literature relevant to this review was completed in October 1961. References to work published before 1960 have been reduced to a minimum since these can be found in the later papers cited here.

² The following abbreviations will be used: NADH₂ (nicotinamide-adenine dinucleotide, reduced form).

vitamins A (9); the relationships between vitamin A and protein intakes (10); vitamin A in mucopolysaccharide biosynthesis (11), in carbohydrate metabolism and adrenocorticoid production (12), and in lipid metabolism (13); the pathology of vitamin A deficiency (14); the role of vitamin A acid (15); and a general discussion (16). Together, these papers provide a wide and valuable survey of recent work on vitamin A.

The role of vitamin A in vision can be largely explained at the molecular level, in terms of the work of Wald and Hubbard on the *cis-trans* isomerization cycle of retinene, concerning which further reviews have appeared (9, 17, 18). Although the general picture has been established and accepted, work has continued on the many minor problems raised. Pullman & Pullman (19) have discussed why the 11-*cis* isomer,³ which is functional in the eye, can be formed so readily despite being a sterically hindered form (18). Several workers have tried to clarify the steps in the breakdown of rhodopsin by light (bleaching). In the scheme originally proposed by Wald and modified by Hubbard & Kropf (20), rhodopsin was converted by a photochemical reaction to lumirhodopsin, which broke down to retinene by thermal reactions. Investigations by other workers, using flash-photolysis (21, 22) or low temperatures (23, 24), or studying the ultra-violet spectrum of rhodopsin (25), agree broadly with this picture, but indicate that it is rather more complicated than it was at first thought (20). The results of Bridges (26), too, are hard to explain in terms of the Wald-Hubbard hypotheses. Hubbard & Kropf (27) see no need to revise their interpretation of the mechanism of the bleaching reactions, but concur that it is incomplete as there are almost certainly further intermediates (22, 23, 24).

There is clearly much to be learnt about the bleaching and synthesis of visual pigments in solutions prepared from eyes, but the experimental conditions (at present obligatory) are quite unphysiological, and may not lead to results which apply to events in the living eye.

Although some workers disagree (23, 24, 26) with the nomenclature and mechanisms proposed by Hubbard & Kropf (20) the matter must be kept in perspective. In fact, the role of vitamin A in vision is better understood by far than any other major function of the fat-soluble vitamins.

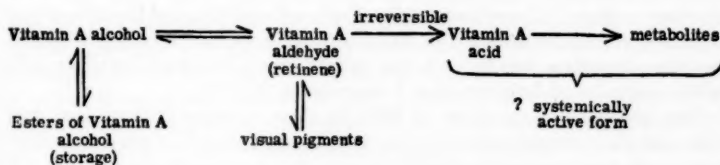
Dowling has studied further the movements and interconversions of retinene, vitamin A alcohol, and its esters in the rat eye (28), and has surveyed his previous work on the effect of vitamin A deficiency in vision (15, 29, 30). Rhodopsins have been isolated from more crustacea (31) and Wolken *et al.* (32) have found retinene in housefly heads, thus adding to the now considerable body of evidence that the retinene system for vision is the general one in Nature.

³ The numbering system used for vitamin A and retinene is the same as the official carotenoid system, i.e., carbon atom 1 has the *gem*-dimethyl group, and the terminal carbon bearing the primary alcohol group is 15.

Knowledge of the other activities of vitamin A is much less satisfactory. Dowling & Wald (15), using vitamin A acid, brought out clearly a dichotomy between mediating vision and subserving the "systemic" role of maintaining life and growth, the health of epithelial tissues and of the body as a whole. Vitamin A acid was completely ineffective in the visual role, as it cannot be reduced to the aldehyde, retinene, yet it had high potency in promoting growth. Subsequent investigations (5, 33 to 40) have shown the acid to be effective in various aspects of the systemic role of the vitamin, but it would not support reproduction (41, 42). In rats on a diet otherwise deficient in the vitamin, vitamin A acid maintains normal growth, but spermatogenesis ceases (41); females have a normal oestrus cycle and become pregnant, but resorb their litters and never carry them to term (42).

This seems strong evidence that vitamin A must have at least three roles: in the visual pigments (as retinene); in reproduction, where vitamin A alcohol will suffice, but vitamin A acid will not (perhaps here, too, the aldehyde may be required); and the "systemic" mode of action, i.e., all the other activities which are satisfied by vitamin A acid.

Vitamin A acid is more effective than vitamin A alcohol in inducing hypervitaminosis A (5, 30, 37, 38), a fact in favour of the hypothesis (15, 43) that vitamin A acid, or some compound derived from it (16), is the form of the vitamin active systemically, rather than vitamin A alcohol itself. If so, the normal metabolism of vitamin A may be summarized as below:



Wolf, Johnson, Wright, and Varandani (8, 44, 45) tried to trap vitamin A acid as a metabolite of vitamin A alcohol but failed even to detect it. It has been the experience of many workers that it is extremely difficult to find traces of vitamin A acid in the intestinal tissues even after feeding huge doses of it, unless it be given in the esterified form. Even then only very small quantities are found (35). In contrast, Dmitrovskii (46) has reported that, after feeding a rat 20 mg retinene, he was able to isolate vitamin A acid (0.6 mg %) from the jejunum together with vitamin A alcohol (4.2 mg %).

Although the inability to trap vitamin A acid (8, 44, 45) tells against the hypothesis detailed above, it does not disprove it. There is no doubt that vitamin A acid can be formed by the liver, by means of an enzyme which oxidizes retinene (46, 47). This liver aldehyde dehydrogenase is sensitive to

steroid hormones *in vitro*, e.g., progesterone, testosterone, and androsterone inhibit it, while oestrone and cortisone stimulate it (47, 48). Since it is not certain that the oxidation is physiologically important, or even that the hormones have the same effect *in vivo*, the biological significance of these enzyme-hormone interactions is problematical.

Two productive lines of research have stemmed from the earlier work of Fell & Mellanby (49). Addition of excessive doses of vitamin A to tissue cultures of limb-bone rudiments brings about losses of sulfate and mucopolysaccharide. From this and subsequent work, Wolf & Varandani (33) were led to the hypothesis that vitamin A regulated the formation of mucopolysaccharides, with an excess of vitamin A leading to an increase in the mucus type, and a deficiency of vitamin A leading to an increase in the connective tissue type of mucopolysaccharide. To test this hypothesis they looked for a mucus-forming tissue and elaborated *in vitro* systems of colon segments or homogenates from rats (33) and pigs (50) which would build labelled sulfate or glucose into mucopolysaccharides. In preparations from vitamin A-deficient animals, the incorporation of $^{35}\text{SO}_4$ and glucose was markedly lowered, relative to controls. Addition of vitamin A alcohol, retinene, or vitamin A acid to these systems *in vitro* restored incorporation to normal. Sulfate utilization could also be lowered or abolished by adding lipoxidase to destroy vitamin A, and then be restored partially by adding more vitamin A (33, 50). By using homogenates of rat colon it was shown that the stage in the biosynthesis of mucopolysaccharides that required vitamin A was the activation of sulfate to give 3'-phosphoadenosine-5'-phosphosulfate, and that the defect could be corrected by addition of vitamin A *in vitro*. Once the phosphoadenosine-phosphosulfate was formed, the sulfate group was transferred equally well by normal and deficient colon homogenates (51, 52).

The level of incorporation of $^{35}\text{SO}_4$ in these systems is quite low, but Wolf and his co-workers have taken many precautions to ensure that they have a cell-free system in which it is possible to demonstrate directly the effect of vitamin A (11). The lowering of mucopolysaccharide-bound hexosamine in the colons of deficient rats, relative to controls (53), is as might be expected if such a system were affected by vitamin A deficiency in the living animal.

Grangaud & Nicol (54) have confirmed that vitamin A increases the incorporation of sulfate into mucopolysaccharides by colon segments, but they found the addition *in vitro* of progesterone to be even more effective. The system used by Grangaud & Nicol is cruder than the enzyme preparation of Wolf and co-workers (33), but further reports on this effect of progesterone will be awaited with interest.

Fell has pursued another aspect of her earlier work—the disappearance of metachromasia (a differential staining here due to chondroitin sulfate) from limb-bone rudiments in tissue culture in the presence of vitamin A. This indicates that the cartilaginous matrix is broken down rapidly (49). The

phenomenon is not peculiar to tissue cultures, as similar changes are seen in the cartilage of rabbits dosed with excess vitamin A (55). Like effects can also be produced by the protease papain both on limb-bone rudiments in organ culture (56) and on the cartilage of rabbits when injected *in vivo* (55). Fell & Thomas (49, 56) put forward the tentative hypothesis that vitamin A enhances the activity of a number of cellular enzymes, one of which resembles papain. In excess this would degrade the structural protein component of the cartilage matrix and thus result in a loss of mucopolysaccharide.

Lucy, Dingle & Fell (57) have shown that in normal embryonic cartilage there is a proteolytic enzyme which can remove from the cartilage matrix most of the amino sugars, presumably by attacking the protein to which the mucopolysaccharide of the cartilage is attached; this activity can also be released by hypo-osmotic treatment. The effects of this enzyme or group of enzymes are very like those produced by excess of vitamin A on limb-bone rudiments (58).

Dingle (59) has shown that vitamin A has a direct action on isolated particulate components of liver cells (probably the lysosomes), setting free a protease with properties similar to those of the enzyme liberated from cartilaginous rudiments (57). Vitamin A has no effect on the protease once it is liberated. Dingle also mentions (59) preliminary observations indicating that the vitamin has a similar effect on the particulate components of homogenates of embryonic cartilage.

These results are taken to indicate the mechanism by which the vitamin can act at a subcellular level to alter drastically the composition of extracellular material in the cartilage matrix. Further work is needed to show how specific this effect may be, for by irradiating lysosomes with ultraviolet light a bound protease can also be released, a process that can be retarded by pretreating the animals with cortisol, which also protects cartilaginous limb-bone rudiments *in vitro* against excess vitamin A (60, 61).

Although this work (57, 59) has been concentrated on a protease, subcellular particles which contain proteases are also known to contain other enzymes, and the action of vitamin A on cartilaginous rudiments in culture causes a complex series of changes including loss of ribonucleic acid (58), so the vitamin probably releases further enzymes.

This hypothesis of the mode of action of vitamin A bears a superficial resemblance to one put forward in connection with vitamin E and muscular dystrophy (62) and will no doubt need refining.

Both the effects of vitamin A so far discussed—the release of a protease, and a stimulation of mucopolysaccharide synthesis—can be demonstrated at the same time in *Xenopus* larvae (63). When one considers these effects, together with the finding that the addition of vitamin A to *in vitro* preparations from deficient animals will restore the conversion of deoxycorticosterone into corticosterone (12, 36, 52), it appears that either the “systemic” role of vitamin A covers a number of quite separate functions or some of these

experimental findings are irrelevant to the more direct physiological action of the vitamin.

An important fact about Fell's work is that it concerns hypervitaminosis A, which probably involves more than just an exaggeration of a normal physiological function. As for the reactivation of enzyme systems by adding vitamin A *in vitro*, it must be admitted that this approach is under a slight cloud at the moment as a result of some chastening experiences with tocopherol and other lipids in oxidizing and phosphorylating preparations. This misgiving may be unwarranted, but if it is true that vitamin A is required for activating sulfate, the enzymic system affected must be restricted to certain tissues, as vitamin A-deficient pigs showed an increased sulfate uptake into some parts of the body (64).

If it were possible to put a finger on one or two categorically fundamental manifestations of vitamin A deficiency, many interesting and significant interrelationships might be seen to reflect secondary phenomena.

The conversion of β -carotene to vitamin A continues to receive attention. Olson (65, 66) has made a thorough direct study of the absorption and cleavage by rat intestine of physiological doses of ^{14}C - β -carotene. It was converted into vitamin A ester in the intestinal wall, and small amounts of retinene, vitamin A alcohol, and unidentified products were also formed. His results did not enable him to make a final choice between the two mechanisms which have been suggested for the conversion, i.e., central cleavage to give two molecules of vitamin A, or terminal splitting, followed by a stepwise breakdown to give one molecule of the vitamin (6), but they favored the former. Glover, the author of the latter hypothesis, thinks that the terminal split is at most a minor pathway (67). Small quantities of the suggested intermediates have, however, been detected in intestinal epithelium (68).

It is now universally accepted that dietary β -carotene is converted to vitamin A mostly in the intestinal mucosa but there is also evidence—summarized by McGillivray (69)—that other organs can carry out the same process, the lung being particularly mentioned. Olson *et al.* (70) tried to study any such conversion by using radioactive β -carotene in a dog heart-lung system, but effectively no vitamin A was formed. McGillivray (69) has obtained evidence that intravenously injected carotene is converted into vitamin A consequent upon relatively nonspecific reactions initiated in the blood immediately after injection. The most probable explanation is that the carotene undergoes rapid coupled oxidation with unsaturated lipids (probably akin to the general unspecific breakdown of carotenoids in many situations *in vitro* and *in vivo*) and that some of the products are converted to vitamin A. Reports by Grangaud and colleagues (71, 72) that the diacetate of astaxanthin can be converted to vitamin A *in vitro* by rat eyes rest upon somewhat equivocal identification of vitamin A.

Much work has been published, particularly by Ganguly and co-workers on vitamin A esterases. Those acting as hydrolases have been studied (73), and in rat and chicken livers it was possible partially to separate vitamin A

esterase activity from cholesterol esterase and the common esterase hydrolyzing short chain esters of simple alcohols (74). The rat intestinal mucosa has both esterifying and hydrolytic activity and more attention is now being paid to the esterification of vitamin A, which involves a different enzyme or enzymes from those used for hydrolysis (75, 76) but surprisingly does not need coenzyme A or ATP (75). The system, unlike that esterifying cholesterol, has no preference for particular fatty acids, provided they have more than ten carbon atoms (77). Nevertheless, the esterification of vitamin A must be connected in some way with that of cholesterol, since feeding cholesterol markedly increases the capacity of the intestine to esterify both cholesterol and vitamin A (76). Although the enzymes concerned with vitamin A do not appear to be very specific in respect of the fatty acids they use, the esterified vitamin A occurs in rat blood and intestinal muscle mainly as the palmitate, and in the liver exclusively as the palmitate, regardless of the dietary fat composition. Mahadevan & Ganguly (78) think this is due to the selective binding by lipoproteins of the palmitate.

Work continues on the fate of dietary *cis*-isomers of vitamin A (79, 80, 81). The 11-*cis* and 13-*cis* forms have been shown to be converted to some extent to all-*trans* in the gastrointestinal tract, and absorbed and stored in the liver as a mixture of isomers. All three isomers appear to be well absorbed from the intestine; differences here are not responsible for the variations in biological potency. Once in the liver, the stored 11-*cis* isomers are converted to all-*trans* vitamin A (82), a conversion which can be carried out in homogenates (83).

Work on the proteins which carry vitamin A in the blood (84) is of more than academic interest. Children with kwashiorkor have extremely low serum levels of vitamin A, even when adequate amounts of the vitamin are present in the diet and liver stores, owing to lack of the carrier protein for vitamin A (85, 86, 87, 88). A similar phenomenon has been observed in experimental rats (86) and pigs (89).

A number of apocarotenals have been isolated from many plant sources and also from intestinal epithelium (68). Some investigations had already been made on these compounds (6, 67) but they have been extended (90, 91, 92) as apocarotenals promise to be useful coloring materials for the food industry. During the search for them in nature, retinene was also found widely in plants and in animal livers (68, 93). This was surprising and probably indicates that retinene complexes (94, 95) are common in plants and animals. Their structure and biological significance remain unknown.

VITAMIN D

The primary action of vitamin D is to increase absorption of calcium from the intestine, and the process can now be studied *in vitro*, using segments of small intestine which transfer calcium ions from the mucosal to the serosal side against a concentration gradient (96, 97).

Active transport in these intestinal sacs is influenced by the vitamin D

status of the animal from which they were obtained; sacs from deficient rats are much less effective than those from animals irradiated with ultraviolet light or dosed with vitamin D. Ergocalciferol, cholecalciferol, and even dihydrotachysterol are equally effective. Addition of the vitamins to the sacs *in vitro* has no effect; prefeeding to the intact animals is necessary (98).

Active transport is most marked in segments from the upper one-fifth of the intestine (96, 97) but it increases when demands for calcium retention are high, e.g., during growth and pregnancy (97), or when the dietary calcium is low; in the latter case, the whole of the intestine can carry out active transport of calcium (99). For this adaptive response, vitamin D is required (99).

The active transport mechanism in these rat intestinal sacs appears to be limited to calcium and strontium ions. It is very much more effective with calcium (99, 100), but it is presumably by means of this process that vitamin D, when given in large doses, can increase the absorption of strontium (101, 102). The active transport mechanism is dependent on oxidative phosphorylation (96, 99, 100) and Schachter *et al.* (100) were able to show the enhancing effect of vitamin D on calcium absorption only in systems carrying out oxidative phosphorylation.

Vitamin D increases the citrate concentration in the serosal medium but this is not the major means by which calcium transport is increased, since the extra citrate could not account for more than a fraction of the extra calcium (96, 98).

The effect of vitamin D can also be shown on rat intestine cut into slices, which for many purposes are more convenient to work with than segments. Slices from normal rats accumulate much more ^{45}Ca from the medium than do those from vitamin D-deficient animals (103). The process has been developed into a new bioassay method (103) and it shows many of the characteristics of the active transfer of calcium by intestinal sacs; it is greatest in the upper small intestine, dependent on oxidative phosphorylation, relatively specific for calcium, and dependent on dietary vitamin D and the age of the rat (103). These studies indicate that the actual accumulation of calcium by the small intestinal wall is affected by vitamin D.

There is even more than this to the action of vitamin D in increasing calcium absorption. By studying the effects of various sugars on the absorption of calcium by intestinal sacs and slices, Schachter *et al.* (99, 100) deduced that active transport covers not only the uptake of calcium ions at the mucosal surface but also their efflux at the serosal surface. Vitamin D clearly stimulates mucosal uptake and the evidence suggests that it also increases serosal efflux.

Harrison & Harrison (104), too, have studied the transfer of calcium *in vitro*, using a slightly different preparation of intestinal loops. They came to the conclusion that vitamin D stimulates not only active transport of calcium, but also its simple diffusion through the intestinal walls. They find that oxidative phosphorylation is not necessary for the latter effect to be

shown and that all segments of the intestine respond to vitamin D in this way.

There is some disagreement between the results obtained by Harrison & Harrison (104) and those of Schachter and co-workers (99, 100) who, finding that vitamin D increases the active transport of calcium even with very high concentrations of calcium in the medium, concluded that its effect on active transport is not due to increasing the permeability of the intestinal wall.

Many features of the active transport process, as studied *in vitro*, agree with what is known of calcium absorption *in vivo* (97) and, on balance, it seems more probable that the primary effect of vitamin D is on the active transport mechanism rather than on the permeability of the intestinal wall. But it is difficult to be certain about the physiological importance of the former process as the rat's requirements of vitamin D appear to be extremely low, and it is very difficult to induce frank rickets even on a diet devoid of the vitamin. It may be that the important function of vitamin D in the rat is to increase the facultative active transport mechanism in conditions when calcium needs are great or dietary calcium is low (98).

In the chick, which becomes rachitic on mere exclusion of vitamin D from the diet, similar experiments were unsuccessful. Isolated loops of chick duodenum showed neither active transport nor response to cholecalciferol (105). This is only a failure to reproduce *in vitro* an action of the vitamin which can be demonstrated in the living bird: calcium disappears from tied-off duodenal loops *in vivo* much more quickly in chicks given cholecalciferol than in rachitic birds (105, 106). Although not yet proven so directly, it seems probable that vitamin D also increases to a lesser extent the absorption by the chick of other metals—strontium, barium, magnesium, beryllium (107), zinc, and cadmium (108). Vitamin D may have a wider role than has hitherto been thought in improving intestinal absorption of metals.

To show its effect on chick duodenal loops *in vivo*, cholecalciferol had to be fed several hours previously (105). In the rat, too, although the active transport mechanism can be fully restored within an hour by feeding huge doses of the vitamin (97), physiological doses take two or three days to produce an effect (100). Restoration of active transport of calcium involves more than merely the accumulation of the vitamin as such in the intestine, since the two effects do not coincide (100). It seems that the action of the vitamin is indirect: either it produces some change in the intestine which takes some time to develop, or it has itself first to be converted into an active form.

Vitamin D must have some other activities besides promoting the absorption of calcium, since dietary calcium deficiency does not produce the same effect as a lack of the vitamin. Only brief reports (100, 109) have appeared of the effect of vitamin D on phosphate transfer in intestinal sacs of rats; it does appear to have some effect but it is probably minor compared with its effect on calcium ions.

Radioactive ergocalciferol continues to be used (110, 111) in an effort to trace these other modes of action. When incubated with rat serum, ergocalciferol becomes attached to the albumins and α_2 -globulins (112), but when ergocalciferol is given orally, the blood does not retain much; it is largely transferred to the tissues (109, 111).

One interesting feature of this work has been that the radioactive ergocalciferol found in the kidney is localized in the proximal convoluted tubule (110). This is the region of the nephron which is usually associated with reabsorption of phosphate, but an effect on calcium should also be considered, as vitamin D can be shown to increase the renal tubular absorption of calcium in dogs (113).

In a review of the nutritional and physiological interactions of calcium and phosphate, Wasserman (114) makes the rather disillusioned comment, the function of vitamin D in the metabolism of calcium and phosphorus has been studied extensively, and it seems that any unqualified statement for vitamin D action can find proponents and opponents in the literature and among researchers currently engaged on the problem.

This exaggerates the lack of unity but it is not absurdly wide of the mark.

In work on intact animals it is difficult to separate primary actions of the vitamin from the ramifications of any change in plasma and tissue levels of calcium and phosphate. It has not been possible to obtain *in vitro* systems in which the variables can be closely controlled, with the exception of the intestinal preparation discussed above. One report (115) mentions that purified pig heart aconitase is inhibited *in vitro* by the addition of large amounts of ergocalciferol and, more effectively, of dihydrotachysterol. The hypercalcaemic effect brought about by these substances when fed in large doses might be due to the inhibition of aconitase and the consequent accumulation of citrate (115), but at present the physiological action of vitamin D cannot be explained in terms as simple as this.

VITAMIN E

The biochemistry of vitamin E has been surveyed by Vasington, Reichard & Nason (116), with particular emphasis on its functions. They deal with most of the topics mentioned in this review and with some other aspects which will not be discussed here.

The hypothesis that the primary action of vitamin E is to act *in vivo* as an inhibitor of lipid peroxidation is still very popular; it has been reviewed by Horwitt (117). In attempts to verify this hypothesis, measurements continue to be made of lipid peroxides, i.e., thiobarbituric acid reactants, and many more reports (118 to 132) have appeared that these are higher in various tissues of animals on vitamin E-deficient diets than in those of controls.

The numerous demonstrations that feeding synthetic antioxidants can cure many vitamin E deficiency symptoms must surely imply that tocopherol

exerts an antioxidant role *in vivo*. Tappel & Zalkin have followed up the hypothesis that the role of tocopherol is to prevent lipid peroxidation catalyzed by haematin compounds (118, 119, 133). Using artificial mixtures, they found synergistic systems with tocopherol to be very effective in inhibiting the peroxidation of linoleic acid catalyzed by haemoglobin. They have explored the notion that a series of coupled oxidation-reduction systems could act *in vivo* to prevent haematin-catalyzed lipid peroxidations (133).

If tocopherol fails to prevent such peroxidation, Tappel & Zalkin believe that its impact may be particularly damaging to structures rich in lipoproteins, e.g., mitochondria and microsomes. Using electron transport particles *in vitro* they have been able to show a close correlation between lipid peroxidation and a decrease in the rate of oxidation of succinate (133). These studies are interesting, but do not clarify the conflicting findings on oxidative systems in tissues from vitamin E-deficient animals (116, 118, 134, 135).

Liver homogenates from deficient animals do however show two well-verified effects on oxidative systems. The first is an impaired synthesis of ascorbic acid due to inhibition of the microsomal enzyme, gulonolactone oxidase (120, 134, 135); the second, an accelerated decline in the oxidation of α -ketoglutarate, succinate and other substrates, which is referred to as "respiratory decline" (118, 136, 137). Both can be overcome by previously feeding α -tocopherol to the animal.

The amount of gulonolactone oxidase present in E-deficient preparations is unaltered, but an inhibitor appears in the microsomal fraction. It can be produced nonenzymically by agents, such as metal ions or ascorbic acid, which cause peroxidation of the microsomal lipids and it can be prevented by the addition *in vitro* of substances such as α -tocopherol which hinder lipid peroxidation (120). Gulonolactone oxidase seems especially sensitive to lipid peroxidation; of a number of microsomal enzymes investigated, it was the only one affected (135). The inhibition can also be brought about by the enzyme itself working on its substrate, gulonolactone, to give ascorbic acid or, perhaps an intermediate between gulonolactone and ascorbic acid (135) which reacts with microsomal lipids to produce an inhibitor (120). This process, too, can be stopped *in vitro* by adding tocopherol and other substances which prevent the peroxidation of lipids (120). Attempts to identify the inhibitor thus produced by the enzyme have been unsuccessful, for there is always a lag period before inhibition is seen. The inhibitor cannot be transferred from one system to another—perhaps it has only a transitory existence, e.g., a free radical—but its precursor accompanies the phospholipids on fractionation of tissue lipids. The inhibitor can be shown to be different from fatty acids oxidized by exposure to oxygen, although these, too, can inhibit gulonolactone oxidase (134, 138).

The rapid decline of oxidation of α -ketoglutarate and succinate in liver homogenates from E-deficient animals is similarly brought about by an inhibitor which is mainly in the microsomal fraction (136, 137, 139). Like

the inhibitor of gulonolactone oxidase it is difficult to extract from the microsomes and may have only a transitory existence (137).

Respiratory decline has some points in common with the inhibition of gulonolactone oxidase. In particular, the inhibitor produced by gulonolactone oxidase in the presence of its substrate also accelerates the decay of succinate oxidation in E-deficient mitochondria (140). This is probably not a major factor in causing respiratory decline, which Corwin & Schwarz (137, 141, 142) believe to result from damage to sulfhydryl groups, for the inhibitor produced by gulonolactone oxidase does not affect SH-containing enzymes (134, 135).

There appear to be other features of respiratory decline which differ from the inhibition of gulonolactone oxidase. These features, unlike the gulonolactone oxidase inhibition, cannot be prevented by the addition of α -tocopherol *in vitro*, even though tocopherol stops lipid peroxidation (143). Conversely, the synthetic antioxidant N-N'-diphenyl-p-phenylene-diamine (DPPD), in doses too small to stop lipid peroxidation, prevents respiratory decline (136, 143, 144). Respiratory decline does not go hand-in-hand with tissue "peroxidation" as does the inhibition of gulonolactone oxidase (120).

It is hard to decide even in homogenates to what extent the activity of gulonolactone oxidase and the nonenzymic oxidation of microsomal lipids contribute to the depression of ascorbic acid synthesis and to respiratory decline, and harder still to know what happens in the intact animal. The livers of E-deficient animals may contain less ascorbic acid than is found in controls, but the difference is not great (125). Respiratory decline is characteristic of the latent phase of the liver necrosis found in E-deficiency, but is itself essentially an *in vitro* phenomenon.

There is no direct evidence that either of these enzyme systems is inhibited *in vivo*, and so the physiological importance of tocopherol as a protective agent remains undefined. Nevertheless, these *in vitro* observations provide a striking demonstration of how tocopherol can strengthen the resistance of tissues to harmful conditions.

A brief report from Tappel's group (62) claims that lipid peroxidation in lysosomal membranes releases a number of enzymes including cathepsin, ribonuclease, β -galactosidase, and arylsulfatase, and that these "free" enzymes in the dystrophic muscle of E-deficient rabbits and mice are raised in concentration many times relative to control animals. They suggest that muscular dystrophy is caused by an increase in these enzymes, which might explain reports that certain enzymic activities are enhanced in the vitamin E-deficient animal (116, 134, 145). The hypothesis has some points in common with one put forward to explain the quite different phenomenon of the action of excess vitamin A (59) (see p. 496).

More detailed information should be interesting. Although there is much evidence that tissues extracted from animals deficient in vitamin E peroxidize more readily when exposed to air than do those from controls, it is difficult to relate this process to the deficiency signs observable in experimental animals.

The usual procedure is to incubate a tissue homogenate in air, measuring the production of substances—mainly malonaldehyde—which react with thiobarbituric acid and arise from peroxidation of polyunsaturated acids. Green *et al.* (123, 125, 146) have discussed critically the assumption that the ability of tissues to resist such peroxidation is a measure of functional vitamin E status. Many tissues peroxidize equally well whether they come from controls or from E-deficient animals. The extent of peroxidation in different tissues does not go in step with the susceptibility of the tissues to vitamin E-deficiency, or indeed with the amount of tocopherol they contain (123, 146). As measured by the thiobarbituric acid test, many substances with no vitamin E activity have "anti-oxidant" properties (120, 123, 134, 135, 144, 147) and feeding a normal stock diet can raise the amount of thiobarbituric acid-reacting material in healthy rats to the level found in animals on an E-deficient diet (120).

The usual thiobarbituric acid test for peroxidation measures the response of homogenized tissues on exposure to air and is essentially an artifact; it is doubtful if any similar process occurs *in vivo* (134, 143). The relationships between tissue "peroxidation" and deficiency signs are, not surprisingly, tenuous.

There is no doubt, however, that feeding polyunsaturated fat adversely affects the vitamin E status of animals; it has been confirmed for chicks (148, 149, 150), rats (149, 151, 152), calves (153), sheep (154), and man (117, 149, 155) that increasing the intake of polyunsaturated acids raises the needs for tocopherol. Horwitt (149, 155) has pointed out these results imply that special care should be taken of vitamin E requirements in human subjects given the currently fashionable diets high in polyunsaturated fat, although most of the adult population of North America and Britain already have satisfactory blood tocopherol levels (156, 157, 158).

The antagonism between polyunsaturated acids and tocopherol is not as simple as it sometimes appears. Chicks develop encephalomalacia on a diet relatively low in vitamin E when fed some polyunsaturated acids (e.g., linoleic). The incidence of encephalomalacia after feeding linoleic acid correlates well with the incorporation of linoleic and polyunsaturated acids into brain mitochondria (149, 159). The development of encephalomalacia can be accelerated by feeding oxidized fats and fatty acid oxidation products (148, 160, 161) or by injecting linoleate hydroperoxide (162). Trichloroacetic acid extracts of the cerebella of encephalomalacic chicks show damage of the sort which would be expected from oxidation by free radicals (163). These results agree with the hypothesis that encephalomalacia is caused by increased peroxidation arising from high tissue levels of polyunsaturated acids (148, 162). Yet linolenic acid fed in large amounts is also incorporated into brain mitochondria (159) without inducing encephalomalacia (149, 155). The simple peroxidation hypothesis must still be treated with caution as an explanation of chick encephalomalacia (155).

The chick, however, is a rather special case since, provided the diet

contains adequate selenium and not much unsaturated fat, it requires very little vitamin E (164)—for all practical purposes, none—and Bunyan *et al.* (165) were able to show that the chick has extremely low levels of tocopherol in its tissues.

The most cogent evidence that tocopherol acts simply as an antioxidant was the finding that synthetic antioxidants such as N-N'-diphenyl-*p*-phenylene-diamine and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin) will maintain the functions of vitamin E (166, 167).

Green and colleagues are not convinced by this, as their newer analytical methods (168, 169) indicate that the diets given to the animals may have contained some α -tocopherol (123, 146), so the synthetic antioxidants could have been sparing the vitamin.

Using these methods, Green and co-workers have been able to determine the amount of tocopherol present in many tissues of the rat (146) and the rabbit (170). Tissues were drained of tocopherol at varying rates, and the picture in the rat was not the same as in the rabbit. Green believes that local tissue deficiencies of vitamin E may occur in animals to the point of causing death, even though other tissues still contain tocopherol. On this hypothesis, species differences in vitamin E-deficiency signs are a reflection of local tissue deficiencies (146, 170).

In contrast, workers at Urbana have been unable to detect any tocopherol in the tissues of animals maintained for up to three generations on E-deficient diets supplemented with N-N'-diphenyl-*p*-phenylene-diamine (166, 167, 171, 172), and Bieri *et al.* (173) have reiterated that they can find no tocopherol in young rats and chicks on a diet deficient in vitamin E.

There is a clash of opinion on the value of the analytical methods employed, and a collaborative investigation might clarify this important point.

Various other aspects of the antioxidation hypothesis have been discussed by a number of authors (116, 123, 125, 137, 143, 146, 167). There seems no doubt that α -tocopherol acts as an antioxidant *in vivo*, but the evidence does not compel us to conclude that this is its only function.

What action can tocopherol have, other than as an antioxidant? Green and co-workers lay emphasis on the effect of tocopherol in maintaining high tissue levels of ubiquinone (coenzyme Q) (122, 123, 125, 146, 170, 174, 175, 176). Synthetic antioxidants which prevent tissue peroxidation do not affect ubiquinone levels (122, 125), perhaps because of their different intracellular distribution (177). Green's hypothesis shows some resemblance to that of Crider *et al.* (166, 167, 171) who have found a compound X, as yet unidentified, which gives an Emmerie-Engel reaction, but is not derived from α -tocopherol. They suggest that the functions of tocopherol and other antioxidants include the maintenance of tissue levels of X. It will be interesting to know its chemical nature (167).

Any explanation of the mode of action of tocopherol must take into

account the now very large number of reports that feeding selenium can cure some, but not all, vitamin E deficiency signs. This does not necessarily indicate that the two substances have the same action—they could have quite independent functions which happen to produce similar results (143)—but possible interrelationships have been considered by a number of workers (123, 125, 128, 141, 143, 178, 179, 180, 181). A popular suggestion has been that some organic selenium compound has antioxidant activity (132, 180, 181), perhaps a selenoaminoacid or selenoprotein (127, 128), but the evidence is unconvincing; in certain circumstances, feeding selenium can actually increase peroxidation (127, 132, 143). Green and co-workers suggest that the common point of action of tocopherol and selenium is on tissue levels of ubiquinone and that the unequal effects of tocopherol and selenium in the whole animal can be ascribed to their unequal effects on the ubiquinone content of different tissues (123, 125).

Some very promising advances have been made by Schwarz, investigating his earlier discovery that rats on some vitamin E-deficient rations developed necrotic degeneration of the liver which could be prevented to some extent by cystine as well as by a third principle called Factor 3. Schwarz (141, 143, 178) has reviewed the evidence that Factor 3 active substances contain selenium and can be synthesized by animals from dietary selenite. Most of the activity of cystine is now attributed to contamination with traces of selenium, but a vitamin E-sparing action persists in highly purified sulfur-containing amino acids.

Two natural products, α -Factor 3 and β -Factor 3, have been separated, the former having an ED 50 (expressed in terms of its selenium content) of 7 μ g in 100 g diet. It may be an aliphatic seleninic or selenonic acid; diseleno- γ - γ -di-n-valeric acid in its racemic form has an ED 50 (in terms of selenium) only twice that of Factor 3 (141).

The nutritional effects of selenium compounds have been reviewed (141, 181, 182, 183) and the work deserves more space than we can give it.

Various possible metabolites of α -tocopherol have been investigated for biological activity: tocopherylquinone and hydroquinone and the lactone of 2-(3-hydroxy-3-methyl-5-carboxy)-pentyl-3,5,6-trimethylbenzoquinone. [For this lactone Green *et al.* (123, 176) propose the trivial name tocopheronolactone, and tocopheronic acid for the corresponding acid.]

Tocopherylquinone maintains tissue ubiquinone levels and has been found active in the rat gestation-resorption test (176); its hydroquinone was active in the latter assay (184). Previous attempts to show biological activity in tocopherylquinone failed, presumably because the rat is unable to store or transport it effectively (169).

Tocopheronolactone is more active than α -tocopherol in keeping up tissue ubiquinone levels and exerts its effect more quickly (123, 176); it is also more potent *in vitro* than α -tocopherol in preventing respiratory decline, but menadione too shows activity and this may be a relatively unspecific effect

of quinones (136, 137). Tocopheronolactone had no activity in the rat gestation-resorption test, or on erythrocyte haemolysis (123).

Green *et al.* (123, 176) have suggested that the active form of vitamin E is tocopheronic acid or a derivative. They have found an enzyme in liver which will oxidize reduced pyridine nucleotides, reducing tocopheronolactone (185), but it is not specific to tocopheronolactone, and no conclusions can be drawn about its significance until the many reports of pyridine nucleotide-quinone reductases are systematized. The failure of tocopheronolactone to prevent foetal resorption, although in the circumstances not conclusive (123), does tell against the suggestion that it is the main active form. A more cogent objection is provided by the work of Alaupović *et al.* (167), who found no tocopheronolactone in the livers or other tissues of rats and pigs after feeding radioactive α -tocopherol. Nor could they detect tocopherylquinone, hydroquinone, and tocopheronic acid, but they did find three new metabolites which are as yet unidentified. Tocopheronolactone and tocopheronic acid are known to occur in the urine of rabbits (186); it seems there may be species differences in the metabolism of tocopherol.

A further substance reported to have vitamin E activity is ubiquinomenol, which can maintain pregnancy in the rat (187). It is difficult to know how much weight to place on reports of vitamin E-active substances given the suspicion voiced by Green (123, 146) that substances with antioxidant properties, such as ubiquinomenol, may work by sparing tocopherol. Full critical assessment may have to wait on the use of a completely tocopherol-free diet, but the evidence does not yet warrant the replacement of α -tocopherol by one of its metabolites as the functional form of vitamin E in tissues, or by a general antioxidant, even though these substances display some activities of the vitamin.

Nason's hypothesis that tocopherol is a co-factor in the cytochrome-c reductase system (116) continues to attract critical papers which indicate that his reactivation of iso-octane-treated preparations includes a nonspecific physical phenomenon (188, 189, 190), but Nason, while admitting this, still believes there is enough evidence to support his hypothesis (116). Slater *et al.* (191) have published details of the careful investigations by which they prove that the substance in Keilin-Hartree heart muscle preparations once thought to be α -tocopheryl quinone is ubiquinone. They find almost no tocopheryl quinone in the preparation, but they confirm that it contains α -tocopherol in about the same amounts as some components of the respiratory chain. The evidence is not conclusive either way, but at present the involvement of tocopherol in the respiratory chain must be considered unproved.

VITAMIN K

A formidable volume of work has been published on vitamin K as an electron carrier (usually in the form of added menadione) and as a possible participant in oxidative phosphorylation. The topic is discussed elsewhere in

this volume, and so will not be pursued further here. A majority of workers regard the physiological importance of vitamin K in oxidations and phosphorylations as slight or nonexistent in animals, although this may not be so in some micro-organisms.

Others, notably Martius (192, 193, 194), oppose the view. His school has carried out much work on the conversion of various members of the vitamin K family to vitamin $K_{2(20)}$ (i.e., 2-methyl-1,4-naphthoquinone, with a 20 carbon atom isoprenoid side chain at position 3) (192, 193, 194). Vitamin K_1 has its side chain removed in the animal body and is turned into $K_{2(20)}$ [some preliminary work is reported on the fate of the phytol side chain (195, 196)], and other members of the K_2 family, $K_{2(30)}$ and $K_{2(10)}$, are also converted in varying degrees to $K_{2(20)}$ in birds and animals (193, 196, 197). The enzyme which adds the side chain to the naphthoquinone nucleus can be obtained from mitochondria; it uses pyrophosphate esters of isoprenoid alcohols to give the K_2 series. The enzyme appears to be the same as that which puts the side chain on the ubiquinones (198, 199); the latter is a more active process, and is probably the major function of the enzyme.

The significance of the formation of $K_{2(20)}$ in the animal, and indeed the possibility of its participation in oxidative processes, rests on whether any form of the vitamin normally occurs in mitochondria. As revealed in the discussion of a paper by Martius (192), workers are sharply divided on this issue: many cannot detect the vitamin there (200, 201). The analytical problem is severe, but it should not be insoluble. Some quite valuable results have been obtained with the admittedly much easier case of the bacteria (202, 203, 204). Until it can be shown whether vitamin K is or is not a normal and necessary constituent of mitochondria, many problems will remain in an unsatisfactory state of controversy.

Whatever the truth about the postulated role of vitamin K in electron transport and oxidative phosphorylation, there can be no denying that it is necessary for the formation of some part of the prothrombin complex. Bearing in mind the wide distribution of the vitamins K in nature, Martius (194) regards the decline in prothrombin synthesis as just the *locus minoris resistentiae* in vitamin K-deficient animals, which seems very likely since the vitamin is a growth factor for rats (205). He suggests (192, 193, 194) that the primary defect in K deficiency is in oxidative phosphorylation.

By using 2-chloro-3-phytyl-1,4-naphthoquinone, which in intact animals acts as a competitive antagonist of vitamin K in blood coagulation (206, 207), the prothrombin time in rats can be increased to over 15 times that of controls, without changing the P:O ratio of liver mitochondria (208). Similarly, $NADH_2$ -oxidase activity and P:O ratios remained unchanged in rats fed K-deficient rations which induced prolonged prothrombin times (209). These findings can be used as arguments against the hypothesis that vitamin K plays a role in oxidative phosphorylation; in this respect they are not conclusive, but they seem to negate the possibility (192, 193, 194) that

the defect in prothrombin synthesis is a consequence of faulty oxidative phosphorylation.

Changes in oxidative enzymes have been reported in animals made vitamin K-deficient by feeding beef which had been exposed to ionizing radiation (209); the cytochrome oxidase activity of many tissues rises and is lowered again to normal by feeding vitamin K; liver mitochondria show an increase in dinitrophenol-stimulated adenosine triphosphatase. The hemorrhagic syndrome and the raised cytochrome oxidase level go together to some extent, but the correlation is imperfect (209) and wider conclusions cannot yet be drawn from this work.

A fairly clear picture has now emerged from the work carried out in the last few years on the relationship between vitamin K nutrition and the hemorrhagic lesions caused by feeding irradiated beef to rats. A good account is presented in a review of the work of Johnson's school (210).

The rat requires vitamin K in its diet, but coprophagy can supply some of its requirements. The extent of coprophagy varies with different diets, but it can provide around 5 μ g vitamin K per day (210). If coprophagy is prevented, the rat is dependent on an almost daily intake of the vitamin. Feeding 0.1 μ g vitamin K₁ per g diet satisfied the needs for maintenance of normal plasma prothrombin levels and for growth, which were about equal (205). The relationship between dietary vitamin K level and the prothrombin time indicated that the rat obtains no more than traces of vitamin K by directly absorbing from the gut material synthesized by the intestinal flora (205).

The female rat is much less susceptible than the male to vitamin K deficiency (211). Administration of male hormones or castration of female rats increases the susceptibility to hypoprothrombinemia and hemorrhage; the converse is true for the administration of oestrogens or castration of the male animal (212, 213, 214).

On irradiation of beef, most of the vitamin K is destroyed, but no K antagonist is produced; irradiated beef ceases to induce K deficiency if K₁ is added to it (215). It appears that the presence of irradiated beef in the diet helps to induce K deficiency by discouraging coprophagy (210).

Other dietary components can be shown to influence K deficiency: the feeding of large amounts of vitamins A and E (212), partially oxidized fats or squalene (216) accelerates it; the addition of methionine protects against it (217).

One of the most remarkable features of K deficiency in the rat is that, unlike any other vitamin deficiency, it can be induced within days. Also, it is induced more quickly in the adult rat than in the growing animal (217).

These results obtained on rats cannot be applied to all other species, as cats and dogs are resistant to a K-deficient diet which is lethal to rats (218). No generalizations can be made about the importance or otherwise of microbial synthesis of vitamin K in the intestine pending more information on other species.

Isler and co-workers have extended their previous synthetic work to $K_{2(46)}$ and $K_{2(50)}$ (204). There is some controversy about the vitamin K-type compound which Russell & Brodie (219) have found in *Mycobacterium phlei* and have implicated in oxidative phosphorylation. Noll (204, 220) has reported it to be $K_{2(46)}$ although Brodie disagrees with this and believes it to be a K_1 type (221). There is some support for the latter suggestion (203) but the exact structure is unknown.

Until a few years ago, ideas about the vitamins K to be found in nature were clear and simple. The picture is now more complex, and in the future may be disarrayed yet further by the discovery of more naturally occurring vitamin K compounds.

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NUTRITION—GERMFREE ANIMAL RESEARCH¹

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INTRODUCTION

No area of nutritional research is as interesting and appealing as the work with germfree animals. Part of this appeal arises from the implications of the germfree state. The cost of the apparatus and the painstaking work required to start and maintain a germfree colony have, until recently, limited the activity in this area to a small number of laboratories.

Research programs with germfree animals are in operation at the following centers: Lobund, at the University of Notre Dame; Germfree Life Research Center, Tampa, Florida; Walter Reed Army Medical Center, Washington, D. C.; National Institutes of Health, Bethesda, Maryland; Biological and Food Research Center, Syracuse University; Department of Pathology, University of Michigan; Department of Pathology, University of Arkansas Medical School; Department of Biochemistry, University of Missouri Medical School; College of Veterinary Medicine, Michigan State University; College of Veterinary Pathology, Ohio State University; Laboratory for Germfree Research, Karolinska Institutet, Stockholm; and Department of Pathology, Nagoya University, Japan. Of these, only the Lobund, Stockholm, and Nagoya installations were in operation ten years ago. The advent of economical plastic units (1, 2, 3) in which germfree animals can be kept for a number of months will permit other laboratories to use these animals. A portable unit for transporting germfree animals without contamination (4) facilitates this possibility.

Terminology.—The term "germfree" has its limitations. To some it may imply that the animals are free only of pathogenic or harmful bacteria. This confusion may arise from the association of germfree animals with "specific pathogen-free," "pathogen-free," or "disease-free" animals.² Colonies of these animals have been started either with germfree animals (7) or animals removed by hysterectomy and raised in a clean environment (8). In these cases the gastrointestinal tracts of the animals are inoculated fortuitously with a variety of bacteria that are indigenous to the "normal" animal. No information is available on the types of bacteria present in the gastrointestinal tracts of these animals (9, 10).

The germfree animal has been defined as "a pure animal system free from

¹ The survey of literature pertaining to this review was completed in December 1961.

² Commercial production of specific pathogen-free rats has been plagued by chronic respiratory ailments in the animals (5) in spite of special housing and elaborate control measures (6).

all detectable contaminants" (11). This definition is circumscribed by the tests used for detecting contaminants which are limited in most cases to bacteria and protozoans that are present in the feces, body orifices, and on the surface of the animals. Some attempts have been made to determine the sterility of the entire animal. Early reports from Lobund (12) indicate that after a litter had been introduced into a germfree tank one animal was sacrificed and then minced and a part of the tissue plated on a variety of media. Gustafsson occasionally keeps within the tank for a week or two the animals that have died during the course of an experiment (13); the animal serves as a medium for any bacteria that it harbored. Another investigator uses the whole animal (guinea pig) for bacteriological examination at the end of the experiment (14). These procedures are essential for short-term studies, since certain types of infection may otherwise go undetected.

For rats, mice, guinea pigs, and chicks, it is assumed that when the conventional parents appear healthy and are of "clean" stock there is little likelihood that the germfree offspring will be contaminated, provided due precautions were used in bringing them into the germfree tank (15). Even under these circumstances a careful autopsy of the germfree animal should be performed at the end of the experiment. Hens that appear healthy and of "clean" stock may be carriers of *Salmonella pullorum* (16). This organism may enter the germfree tank, presumably in the egg. Dogs may have been infected with parasites *in utero*. Of the six "germfree" dogs secured at Lobund, nematodes were found in five of them (17).

Only one investigator has reported the presence of what was diagnosed as a viral disease in germfree animals (18). A few reports suggest the presence of the ECHO viruses (19) and an unidentified virus (20) in some germfree rats.

The earlier workers used such terms as "pure" or "bacteria-free" for their germfree animals (21). In 1942, Baker & Ferguson (22) suggested axenic "to denote a living organism that is free from all other demonstrable organisms." Later, Lobund proposed the term "gnotobiotic" to describe the condition in which an animal "is free from contamination or is in association with known organisms" (21). It is derived from Greek words meaning "known life" and covers the spectrum from "germfree animals" to those that are infected with specific organisms.

Although each of these terms has merit, "germfree" will not be abandoned since it is well entrenched in the literature. Animals contaminated with one strain of bacteria are designated "monocontaminated"; those brought out of the "germfree" environment and infected with a normal flora are called "ex-germfree"; those kept in the animal room with a normal flora are conventional controls.

Reviews.—Some reviews of this subject have appeared as introductions to reports of research findings (13, 23, 24); others are historical surveys (11, 25, 26, 27, 27a).

HISTORICAL

Pasteur provided the impetus which led to the study of germfree animals (28). He suggested that an animal could not live in the germfree state (29). Nuttall & Thierfelder (30, 31) initiated experiments which they hoped would disprove Pasteur's statement. These investigators used a glass bell jar inverted in an oil bath overlaying water. Inside the jar, above the oil level, was a wire screen on which the guinea pig was kept. Feces, urine, and other debris fell through the oil layer for removal from the germfree chamber. A guinea pig was removed by Caesarian section and placed in the sterilized apparatus without bacterial contamination. The guinea pig was fed cow's milk secured under "aseptic" conditions and sterilized by heating on two successive days. The animal died after eight days, at which time the intestinal contents and fecal samples were sterile. Since the guinea pig lived for eight days, Nuttall & Thierfelder concluded that animals could live in a germfree state and Pasteur was wrong.

A few years later, these workers tried to raise germfree chicks. Their difficulties led to the suggestion that it was impossible to sterilize the outside of the shell (32). Schottelius (1898) succeeded in starting ten germfree chicks but they did not grow when fed a sterilized mixture of millet, egg white, and egg shells. Later, Schottelius kept a number of germfree chicks for 29 days, but none of these gained very much weight (33). Cohendy (34) had similar results with germfree chicks. He fed conventional chicks the same sterilized diet used for the germfree chicks.

In 1912 Kuster (35) attempted to raise germfree goats. He attached two arm-length rubber gloves to the walls of his chamber, thus permitting the operator to reach all parts. A lock containing an electric heater was attached to one end. The lock had doors, one of which opened to the outside and the other into the chamber. Supplies could be brought into and out of the chamber with a minimum risk of contamination. Air for the chamber was sterilized by bubbling through concentrated sulfuric acid and subsequent passage over a heated coil. The chamber was sterilized with formaldehyde vapors. A gauze hood was built over the lock to cover the pregnant goat during the Caesarian operation. Aseptic technique and speed were the factors which permitted the delivery of a germfree goat into the chamber.

Since the doe had twins, Kuster used the second kid as a control. Although the control was two pounds heavier at birth, it did not gain weight as rapidly as the germfree animal. Both kids were fed milk (presumably secured from the doe) and oat gruel, which were sterilized prior to being put into the lock. While in the lock, the food was again heated. On the 13th day, the germfree goat became contaminated with "hay" bacilli but was maintained in the chamber as a monocontaminated animal for another 22 days.

These pioneering studies indicated that a variety of animals could be kept for periods as long as a month in a germfree state. They answered the question: can higher animals be maintained for periods of a few weeks in the

absence of bacteria? The attainment of this objective terminated further work, even though Kuster suggested that investigations of absorption and digestion, immunological reactions, and wound healing should be carried out with germfree animals (35).

Between 1912 and 1936 little work was done with germfree animals. A brief report appeared in 1922 (36) indicating that germfree guinea pigs developed scurvy and showed symptoms similar to those of conventional animals. The report stated that when the germfree guinea pig was inoculated orally with a culture of "vibron cholérique" it died within 48 hours, showing the presence of large numbers of colera bacilli in the intestine. A conventional guinea pig inoculated in the same way showed no signs of infection and, on autopsy, the intestinal contents and blood were free of cholera organisms.

About 1930, there was an upsurge of interest in germfree work. Two laboratories (Lobund at Notre Dame and the Histology Department at the University of Lund, Sweden) started long-term studies of germfree animals. At the same time an important contribution appeared from the plant physiology laboratory of the University of Warsaw. Balzam's contributions (37) were not adequately evaluated by subsequent investigators. He used a box with a glass top and two glass sides for his germfree chamber. Arm-length rubber gloves were fastened to one of the metal sides. This chamber was attached to another chamber. Access to the latter was by means of a hermetically sealed door which could be opened through another set of gloves attached to the walls of the "ante-chamber." Chick feed was sterilized in a flask plugged with cotton, and on removal from the autoclave was placed in the ante-chamber. There the bacteria in the air were carried down by water vapor generated in a still attached to the ante-chamber by means of a rubber tube.

Balzam reared germfree chicks in his chamber for 59 days, at which time the heaviest male weighed 615 g; two other males weighed 570 and 550 g. Conventional animals fed the same sterilized diet weighed the same as the germfree chicks. Both sets of animals weighed considerably less than conventional birds fed the nonsterilized diet. Since there was no vitamin C in the autoclaved ration, Balzam felt his study provided an answer to a question which bothered some investigators: is the intestinal flora the source of vitamin C in those animals which do not require a dietary source of this vitamin? He observed that the germfree chicks showed the same percentage utilization of their feed as the conventional birds fed the sterilized diet. When germfree birds were fed a ration that was free of yeast they showed typical symptoms of beriberi and developed the deficiency as rapidly as conventional birds. Germfree chicks removed from the chamber and placed in a contaminated laboratory survived with no ill affects. Balzam was impressed by the proteolytic activity of the feces from germfree chicks as evidenced by the liquefaction of the gelatin cultures. This he attributed to the presence of enzymes which presumably were not present in feces from conventional chicks. The feces from germfree chicks had a sweet smell while those from conventional chicks had a sour smell. As a check on the sterility of the birds hatched in his

germfree chamber, he killed one chick shortly after birth and placed it in a sterile broth solution within the germfree chamber.

Glimstedt (38, 39, 40), in the Histology Department of the University of Lund, built a chamber modeled on that of Kuster and sterilized it by passing formaldehyde vapor and steam into it for 48 hours. The pregnant guinea pigs were judged ready to deliver when Glimstedt could insert his index finger between the pubic bones; then he performed the Caesarian operation. The operation was carried out under aseptic conditions in the room where the germfree chamber was kept. The new-born guinea pigs were transferred immediately to the chamber. In spite of the care exercised, the operation and transfer to the chamber were major sources of contamination. Although eight germfree guinea pigs were secured, only three survived for 60 days. They had been fed a sterilized mixture of natural foods. Their weight gain was slightly less than that of conventional animals born and reared under the same conditions.

Glimstedt found the lymphatic tissues of the germfree guinea pigs markedly underdeveloped. This was especially pronounced for the mesenteric, bronchial, and cervical lymph nodes—bacteria appeared to be essential for the full development of the lymphatic tissue close to the respiratory and gastrointestinal tracts. He made only passing reference to the increased weight of the intestinal contents of his germfree guinea pigs. His data show that the intestinal contents of the germfree animals represented 22 per cent of the body weight, while in the controls it was 13 per cent—there was practically no overlapping for the two groups.

In 1928, Reyniers started the work on germfree animals which led to the establishment of the Laboratory of Bacteriology of the University of Notre Dame, more popularly known as Lobund (41). The scope of this operation increased to the point where, at one time, an entire room was maintained as a germfree chamber. Admission was through a formaldehyde vat which was entered only after the operator donned a plastic suit provided with an air supply. By this means, the operator could take care of the animals directly in the germfree room.

About ten years after Glimstedt published his thesis on germfree work, Gustafsson started his germfree work at the same university (Lund, Sweden). He developed a rectangular-shaped stainless steel tank with a window that extended the length of the tank (13).

Shortly after World War II, M. Miyakawa (42) at Nagoya University in Japan devised a unique isolator. After the germfree animals were installed in the isolator, all operations were carried out by mechanical hands manipulated by remote control.

All of the above workers started either with hens' eggs, the shells of which were sterilized with a bactericidal agent, or with pregnant animals from which the fetuses were removed by Caesarian operation. T. D. Luckey (24) is the only one who attempted to use the less soluble sulfonamides for sterilizing the gastrointestinal tract of adult animals. All of Luckey's efforts in this direction proved fruitless.

EQUIPMENT USED IN GERMFREE WORK

There is no universally accepted terminology for the apparatus used to house germfree animals. Reyniers (43) has proposed names for the various units that were developed in his laboratories.

The stainless steel units used at Lobund and most U. S. laboratories resemble large autoclaves attached to mobile bases. A glass viewing port and long rubber gloves attached to the sides of the units permit the operator to see and reach all parts of the inside. These tanks have glass wool filters for removing the bacteria from the inlet air. The unit can be sterilized by attaching it to a steam line. Transfers from one unit to another, or, to and from the outside, can be made through a smaller autoclave attached to one side or the end of the unit.

The surgical unit differs from the above in that it has a false floor through the middle of the tank which contains an opening covered with cellophane. Below this opening is a platform attached to a screw mechanism. When the unit is used for Caesarian removal of young, the pregnant animal prepared for surgery is fastened to the platform which is then raised so that the abdomen touches the cellophane. The Caesarian section is performed from the upper part of the tank after cutting through the cellophane. As soon as the newborn animals are checked to see that they are breathing, they are transferred to the sterile rearing unit through the small autoclave.

The above tanks are commercially available.³ The cost of each unit exceeds \$5,000 (44). The standard rearing unit will house approximately 60 adult mice, or 16 to 20 adult rats, or 4 adult White Leghorn chickens, or 8 adult Bantam chickens (43, 44, 45). Some of these tanks have been maintained sterile for three years with approximately one entry through the autoclave each week (Newton, personal communication).

The rearing unit developed by Gustafsson is of lighter metal than the Reyniers' unit and must be sterilized in a large autoclave (46). A glass plate forms the top of the tank and makes every part of the unit readily visible. The primary characteristic of the Gustafsson unit is its rectangular shape which permits maximum utilization of space. The unit has heated coils for destroying the bacteria and viruses in the air intake.

Both the Lobund group (47) and Gustafsson (personal communication) have developed small units for transporting animals in the germfree state from one laboratory to another. Gustafsson shipped successfully some germfree rats from Sweden to Bethesda, Maryland.

The most impressive germfree tank is that developed by Miyakawa (48). It is equipped for remote control operation and can be joined to a surgical unit by means of an autoclave. Caesarian section is performed in the surgical unit after which the animals are transferred through the previously sterilized autoclave into the main unit. Once in the main unit, the animals are handled by mechanical fingers that are manipulated by means of rods. The operator

³ Reyniers and Sons, 3806 N. Ashland Ave., Chicago 13, Ill. Hickey (44) gives comparative costs of units.

can see what he is doing through the windows in the unit. Miyakawa also uses cylindrical units which are similar to and patterned after the ones developed by Reyniers (42). Each of these units will house 10 guinea pigs (49).

A variety of plastic units have been developed for both short-term and long-term work. Some of these units can be attached to steel entry ports in which sterilization is achieved by peracetic acid (50). Since the acid produces only surface sterilization, diets must be in plastic containers and be pre-sterilized. The insides of the plastic units are sterilized with a peracetic acid mist (1, 2, 51, 52), or, for the rigid plastic units, by washing with a detergent solution followed by an iodine solution and then spraying with formaldehyde (3).

THE TECHNIQUE OF GERMFREE WORK

Starting a germfree colony.—The first workers in this field recognized that germfree animals could be secured either by Caesarian section or from fertile eggs whose shells were sterilized prior to hatching (30, 31, 32, 33, 53).

The Caesarian removal of fetuses can be carried out with minimum danger of contamination in surgical units. The procedure described by Gustafsson (13) for this operation is complex in that the shaved abdominal skin of the pregnant animal is cut and fastened to a ring by means of hooks. The ring is fastened to the lower part of the cellophane-covered opening in the surgical unit. The ring contains fine tubes through which an iodine solution can be passed to sterilize the dermal opening and adjacent apparatus. After the area has been flushed with the antiseptic, the operation proceeds. In the case of the rat, the last two fetuses in the uterus are left in place (13) to reduce the possibility of infection via the vagina. In the case of the guinea pig a hemostat across the distal end of the uterus serves the same purpose (54). Few guinea pigs weighing less than 80 g will survive being reared away from their mothers (27).⁴ After the young are delivered they are wiped dry and then transferred to the rearing unit directly (12, 42) or placed in small sterile bottles closed with rubber stoppers. The latter are transferred through a germicidal trap to the outside and then through another trap to a rearing unit (13).

Theoretically, the guinea pig is the ideal mammal with which to start a germfree colony. Since these animals are born in such an advanced stage of development and start eating stock diet within a day, the problems associated with their rearing are minimal. However, early in life they, as well as other mammals, develop enlarged ceca which appear to be the cause of their premature deaths.

The problems associated with the rearing of germfree rats and mice are compounded by the necessity of hand-feeding the first generation.

Starting a colony of germfree chicks presents relatively few problems. The fertile eggs are washed with a detergent and dipped into a HgCl_2 solu-

⁴ A recent report states that no mortality occurred when the guinea pigs weighed 67 g at the time of Caesarian section (55).

tion. This is usually done on the 18th to 20th day of incubation after the eggs have been candled. The eggs are transferred through a germicidal trap to the germfree tank where the eggs are rinsed with sterile water and incubation is completed (15, 56). These procedures do not harm the developing embryo (57). From hens' eggs treated in the above manner, the hatch is about 75 per cent (58). The temperature in the tank is gradually reduced during the two weeks following hatching from 37°C to room temperature (59). A procedure similar to the above was used to start turkey poults (60) and Japanese quail (56). Although a second generation chick has been obtained (61) it is doubtful whether reproduction within the tank will replace the above procedure.

Rearing through weaning.—For the first few days after mice and rats have been delivered by Caesarian section, they must be cared for on a 24-hour basis. The earlier *ad libitum* technique devised by Lobund (12) was very ineffective (62) and has been replaced by the forced-feeding procedure of Gustafsson (13).

The food is a sterilized mixture of equal parts of cows' milk and 18 per cent cream (62). It is homogenized within the tank by forcing it rapidly through a fine needle in a syringe against the bottom of the container. A fine catheter attached to the needle is inserted through the mouth of the rat into its stomach. Milk is slowly forced into the animal with frequent pauses. The amount of milk required to fill the stomach (0.1 to 0.5 ml) is judged by the area of whiteness on the belly of the young rat. Feeding is repeated every four to five hours (13). After each feeding, the genitals of the animals are gently stroked with a gauze to facilitate urination and defecation. The suckling animals are kept in cages containing soft shavings which were autoclaved twice.

This procedure must be carried out for three weeks. These animals at weaning are smaller than conventional rats, probably as a result of the nutritional inadequacy of the sterilized milk. Gustafsson (13) used a mixture of 15 per cent cream, Aminosol, glucose, vitamins, and salts. The different components were sterilized separately and combined within the rearing tank. Sterilization was associated with a reduction in growth, even in conventional rats (13).

With this procedure, 18 of 25 litters were weaned (62). Even under the best circumstances, the mortality rate is high, especially during the first 6 days (13). Although the rats do not grow at a normal rate, they mature and reproduce. Once this has been achieved, germfree rats can conceive, deliver, and rear their young in the tank. Such animals grow and survive better than those delivered by Caesarian section and then hand-reared (11).

One report indicates that, for the pre-weanling rat, vitamin C is a growth stimulant and is necessary for good health (12). The germfree rats that are hand-fed the sterilized cream, supplemented with a specially prepared casein curd, salts, and lactalbumin, weighed 11.3 g on the 18th day whereas those fed the same diet containing 50 mg vitamin C per 100 ml weighed 16.6 g. Approximately all rats fed the vitamin C-supplemented milk survived the

18 day period, while only 34 per cent of those fed the unsupplemented ration survived. Similar differences in growth and survival were seen in the hand-reared conventional rats. This experiment should be repeated now that better techniques are available for preparing substituted milks, since even with the vitamin C supplement, the weight gain was, at best, only half that of the rats suckled by their dams.

Mice can be reared by the forced-feeding technique, but they are more susceptible to injuries from feeding and handling than are rats. Consequently, only eight of 134 Swiss mice were weaned by this technique. The record with C_3H mice was even worse when only one of 95 males was weaned. Caesarian-born C_3H mice were eventually turned over to Swiss foster mothers and reared by that means (62).

Guinea pigs were reared, from the time of Caesarian section, on a thick slurry of equal parts of a commercial laboratory feed and rolled oats plus small amounts of dextrose and sodium chloride (55, 63, 64). A sterile solution of thiamine and vitamin C was added to the mixture after autoculving. The animals were fed restricted amounts of the ration twice a day.

Germfree guinea pigs lost weight during the first few days and regained their birth weights only by the eighth day. Thereafter they gained about 3 g per day which is considerably less than the 6 to 7 g considered normal (65). The restricted body-weight gain prevented the cecum from becoming to large (63). In another laboratory, guinea pigs were fed a mixture of diluted milk, liver, yeast extract, and vitamins for the first five days after which a semi-purified soybean flour-sucrose ration was added to the mixture (42).

Rabbits have been reared in the germfree state by techniques comparable to those used for rats (62, 66). For the rabbits, the mixture of cows' milk and cream was supplemented with "skim milk protein," amino acids, and vitamins. Apparently little success has been achieved with rabbits, since out of 206, only five were maintained germfree beyond four months of age (66). Germfree rabbits are still available "in limited quantities only"; but now "better than 80 per cent" survive the "unnatural hand-feeding period" (66a). One of the major abnormalities was the enlarged cecum which either directly or indirectly caused death (66).

The above explanation emphasizes a major problem in germfree animal research and opens a relatively unexplored field of nutrition. The requirements of Caesarian-delivered conventional animals are practically unknown. In many respects these animals behave the same as germfree animals (63). The development of cataracts, loss of hair, deformed legs, and enlarged ceca seen in some germfree rats (62) are probably not caused by the germfree state per se but by a nutritionally inadequate ration. Therefore, it would appear desirable to do a considerable amount of work with conventional animals to determine their specific nutrient requirements when fed "synthetic" diets from birth.

Animals reared in germfree state.—The list of animals which have been maintained germfree is gradually increasing. At the beginning of this century, chicks were kept germfree for 29 days (33) and guinea pigs for eight

days (30, 31). Kuster (35) maintained a goat germfree for 12 days. In 1946, the Lobund group (12) reported the birth and rearing of three litters of rats within the germfree tanks. These animals were kept in the tanks for periods of 82 to 115 days. Recent reports (47) indicate that rats have been bred through 14 generations, C_3H mice through 8 generations, Swiss mice through 11 generations and chicks through 2 generations. A number of other laboratories have raised rats through successive generations (67; Levenson, McDaniel & Newton, personal communication). The guinea pig has been bred and delivered in the germfree tanks, but the young females have never survived for more than a few weeks (55, 68). A few second generation male guinea pigs were maintained germfree for as long as eight months when they were sacrificed (McDaniel, personal communication). Rabbits (62, 66), dogs (13), monkeys (69), and sheep (70, 71) have been kept germfree for short periods, but the technical difficulties associated with their rearing explain the absence of any definitive studies.

Controls for germfree work.—Only passing reference has been given by most workers to the question of proper controls for germfree animals. In most experiments, the conventional controls were animals born and reared outside the tanks but fed the sterilized diet offered the germfree animals (42, 72, 73, 74, 75). Gordon (26) suggested that environmental factors (housing, etc.) should be added to the absence of intestinal bacteria as distinguishing characteristics of the germfree state when the controls are reared in the animal room. However, the possibility that conventional control animals kept in germfree tanks might not maintain a "normal" intestinal flora was one reason that those controls were not used more frequently, and another reason was that almost twice as many tanks would be needed. To minimize the change in intestinal flora, a conventional "visitor" was brought into the tank that housed the conventional animals. Every 24 hours, the "visitor" was replaced by another conventional chick from the regular laboratory pens (26). No data were presented for this study other than a comparison of the relative weights (as a percentage of body weight) of a few internal organs. This procedure is not necessary for chicks, since conventional birds raised in germfree tanks for four weeks had the same body weight as those reared in regular brooders and fed the sterilized diet (59). Since germfree chicks are started from eggs, this work appears to establish the validity of using conventional birds raised in regular laboratory pens as controls.

The problem becomes slightly more complex when rats or mice are used. Germfree animals now can be secured from parents that have been maintained in germfree tanks for a number of generations. To minimize a possible genetic mutation, it would appear desirable to remove some of the germfree animals from the tank and use them as controls (13). One difficulty with this procedure is that if older animals are removed from the germfree tank, it may be difficult to establish a "normal" flora in them (Daft & McDaniel, personal communication).

A systematic study of the proper controls for germfree animals is urgently needed. Data should be provided on body weights, intestinal flora, weights

of internal organs, body composition, etc., for conventional animals raised in the animal room and in the germfree tanks. Ex-germfree animals serving as controls should also be compared with conventional animals.

Diets used for germfree work.—Many diets fed germfree animals contain natural feeds such as corn, oats, millet, soybean meal, etc. For guinea pigs, the ration developed by Phillips and co-workers (63) has been used. This aqueous mixture of oats and a commercial stock diet is autoclaved into the germfree tanks (76). Germfree rats have been fed an autoclaved mixture of starch, casein, fat, salts, and vitamins mixed with water to form a cake (67). Starch has been the carbohydrate of choice in the purified-type diets (77) since it does not undergo the "browning" reaction when autoclaved with casein, as does sucrose. The difficulty with sucrose has been overcome by autoclaving a concentrated solution of sucrose and mixing it with the dry ingredients within the germfree tank (78), thus avoiding contamination with such nutrients as vitamin K that are present in some starches (79).

The rations used within the past decade for germfree chicks contained starch, casein, a fat, salts, vitamins, and amino acids such as cystine or methionine and arginine (57, 59).

Since the diets fed to germfree animals are sometimes kept in the tank for a number of weeks, it is imperative to maintain the diet for the control animals under similar conditions. The water present in most germfree rations introduces the possibility of contamination (bacterial, mold, etc.) even when the control ration is kept in the refrigerator. Gustafsson (13) has attempted to minimize this problem by daily removal, from the germfree tank, of the sterilized diet for the controls.

Steam sterilization destroys a variable percentage of certain nutrients. For this reason, some investigators occasionally introduce ampules of sterile vitamins (Seitz filtered) through the germicidal lock and mix this solution with the ration inside the germfree tank (McDaniel, personal communication). The destruction of vitamins can be reduced during sterilization by the use of certain mineral mixtures (59). A sufficiently intense beam of cathode rays has been used for sterilizing diets (55, 80). Although cathode rays produce less destruction of nutrients than steam sterilization (81), most investigators prefer steam sterilization because of its greater effectiveness (11). Ethylene oxide which was proposed for the "cold sterilization" of rations destroys both vitamins and amino acids (82, 83, 84).

From a theoretical standpoint it would appear that the liquid ration developed by Greenstein and co-workers (85) would be ideal for germfree work since it is composed of well-characterized substances and presumably can be sterilized by Seitz filtration. An earlier statement indicated that trouble had been encountered in sterilizing this diet (86); more recently, germfree rats have been raised in the same laboratory on this liquid diet through the age of two and one-half months (Levenson, personal communication). Another major problem associated with this liquid diet is its cost. Not only are the ingredients expensive, but considerable time and effort are required to dissolve some of the minerals and amino acids. This still may be

the diet of choice for investigations of serological reactions in germfree animals. Previous studies have been complicated by the development of immunity reactions to the dead bacteria present in the sterilized rations (87).

Bacteriological checks for sterility.—There seems to be reasonable unanimity as to the techniques used in testing a germfree setup for sterility. In most laboratories, swabs are taken once a week from the oral and anal orifices of the animals. Samples of food, feces, and bedding are also removed. All samples for microbiological testing are inserted into individual tubes, plugged, and then removed through the lock which was previously sterilized.

The above samples are inoculated into a variety of standard culture media. Incubation for periods as long as one month is carried out at room temperature, 37°, and 45° to 55°C with some cultures being made under anaerobic conditions (13, 57, 59, 88). Direct microscopic examinations of fecal samples are also made.

Occasionally when an animal dies, it is left in the germfree tank for a week or two as an additional check on sterility (13); the intestinal contents secured at autopsy (58) and the entire animal have been assayed for bacteria (14). A practical test for contamination is the odor of the exhaust air from the germfree tank. When it is sour or foul-smelling, the tank is contaminated—the absence of odor does not guarantee sterility.

Although complete parasitological examinations are not included in the routine checks, Phillips made such a study of the germfree animals at Lobund (17). The rats, mice, guinea pigs, and chicks were shown to be free of parasites, whereas five of the six "germfree" puppies had one or more types of parasites.

As a check on the completeness of diet sterilization, spore test strips have been placed under the diets during irradiation with the Van de Graaff generator (55, 80) or samples of the diet have been seeded with *Bacillus globigii* and *B. stearothermophilus* organisms (55, 81).

CHARACTERISTICS OF GERMFREE ANIMALS

Nutritional.—One would suspect that the primary difference between germfree and conventional animals would be related to the gastrointestinal flora. For this reason, studies have been made to determine whether the germfree rat requires folic acid, biotin, and vitamin K.

Although one of the earlier attempts to produce a vitamin K deficiency in germfree chicks was unsuccessful (89), the diet used in that work may have contained enough of the vitamin to supply the animals' requirements. Analysis of this diet indicated the presence of 2 to 10 µg of vitamin K per 100 g of diet (90). These low levels of the vitamin probably were sufficient to protect the animals.

A vitamin K deficiency with extensive hemorrhages has been produced consistently in germfree rats fed a purified diet containing starch and casein each extracted with petroleum ether (79, 91). Unless the deficient animals were treated with vitamin K, or contaminated by being brought into the laboratory, they died. Treatment of the deficient germfree rat with 200 µg

vitamin K₁ increased the plasma prothrombin level from a value of 10 to 110 per cent of normal within two hours. When the deficient rats were removed from the tank, the higher the microbial contamination of the atmosphere, the faster was the rate of regeneration of prothrombin; the shortest time for complete recovery by this means was 24 hours. Two strains of bacteria were isolated from conventional rats, each of which when used as a monocontaminant in germfree animals overcame the vitamin K deficiency in 24 to 48 hours (92).

Sodium menadione sulfate has been reported inactive in the germfree rat (67, 79). In support of his observations, Gustafsson refers to the work of Collentine & Quick (93) which indicated that when dogs were treated with Dicumarol, menadione sulfate was much less effective than vitamin K₁ in restoring the prothrombin level.

Luckey and co-workers (89) reported that chicks fed a folic acid-deficient ration stopped growing after about two weeks and died in another week. They also reported (90) that the addition of biotin to a ration deficient in this vitamin, not only increased the weight of germfree rats, but also increased their urinary excretion of folic acid. Since the presence of increased amounts of folic acid in the urine was not verified, it may be advisable to await confirmation of this observation before accepting the investigators' suggestion that this can be explained "only by tissue synthesis of folic acid." Caution is indicated, in view of the report by McDaniel (94) that germfree rats fed a folic acid-deficient diet showed a loss of weight, decreased hematocrit and white blood cell counts, and an increased urinary excretion of formiminoglutamic acid. The latter study, plus the statement by Daft (78) that 29 of 30 germfree rats showed symptoms of folic acid deficiency, make it very doubtful that the germfree rat can synthesize folic acid. In the latter study, sulfonamide added to the folic acid-free diet had no influence on the rate at which the deficiency developed in germfree rats.

The germfree rat fed a biotin-deficient diet lost weight, developed alopecia and a spectacled eye condition (90). These symptoms were reversed by an intramuscular injection of 100 μ g of biotin. An unverified report states that bile of germfree chicks contained 0.24 μ g of biotin per g of bile vs. 0.07 μ g in conventional chicks (24).

The earliest report of a thiamine deficiency in germfree animals was that of Balzam (see Historical). He found that germfree and conventional chicks simultaneously developed a thiamine deficiency when fed a deficient diet. This was confirmed at Lobund (89), where germfree and conventional birds had approximately the same concentration of thiamine in the livers and cecal contents. These values were less than half those of conventional birds fed a complete ration.

A thiamine-deficient diet can be produced by steam sterilization—99 per cent of this vitamin may be destroyed thereby (59). The autoclaved diet was used to produce a thiamine deficiency in rats (95). The germfree rats did not gain as much weight and died earlier than the conventional animals fed the same diet. The addition of procaine penicillin to the thiamine-deficient diet protected the conventional rats but had no influence on germ-

free animals. As in the chick, the thiamine contents of the livers of the germfree and conventional rats were similar but much lower than those of animals fed the complete ration (95).

The symptoms associated with a thiamine deficiency in germfree chicks are confusing. In one and the same paper it is stated (96) that germfree deficient chicks do not develop anorexia and that all germfree and conventional chicks fed a thiamine-deficient ration "exhibited anorexia, lack of growth and ataxia. . . . Many . . . were found with opisthotonos."

A riboflavin deficiency in the germfree chick produced earlier death, while the growth response was similar to that of conventional birds fed the same ration (89). There were no differences in the vitamin contents of the liver and cecal contents of these two groups.

A niacin deficiency in germfree chicks, as evidenced by a cessation of growth after two weeks and death of four out of nine animals, has been reported (89). In this study, no attempt was made to produce a niacin deficiency in the conventional birds—they should show typical oral changes as well as poor growth (97, 98). For this reason it is impossible to determine whether the deficiency symptoms in germfree birds are the same as those in conventional chicks. The livers of the germfree, deficient chicks had a higher concentration of niacin than those of conventional birds fed the deficient or complete ration (89). The latter may reflect only differences in liver size.

Germfree rats fed a vitamin A-deficient ration survived as long as conventional animals fed the same ration (99). The germfree rats showed none of the squamous metaplasia and keratinization associated with this deficiency in the conventional animal. Even the eye and vaginal changes which are used as diagnostic aids in this deficiency were absent in the deficient, germfree rats. The latter, however, showed necrosis of the liver which was sufficient to cause death in some cases; the conventional rats showed no such changes. The adrenals and kidneys of the germfree rats showed histological alterations which did not appear in the conventional animals.

Young germfree guinea pigs, fed an ascorbic acid-free ration, developed scurvy with all its typical symptoms within 28 days (63, 100). Although there was no difference in the rate at which germfree and conventional guinea pigs developed scurvy, it took the conventional animals three times as long to recover when ascorbic acid was given (63).

A vitamin D deficiency in the germfree chick produced a marked reduction in growth apparent from the first day of the experiment (96). A similar growth inhibition occurs in conventional birds fed a vitamin D-deficient diet (101).

Growth rate of germfree animals.—Most reports from Lobund state that male germfree rats grow at a rate approximately three-fourths that of conventional animals fed the same sterilized diet (23, 102, 103). Verification of the smaller size of the male germfree rat raised in that laboratory comes from skull and femur measurements (23). The female germfree rat at Lobund apparently grows at the same rate as the conventional animal (24, 95). The few growth curves that are available for older germfree rats indicate that

they increase in weight at the same rate as conventional animals when fed the same autoclaved ration (46, 67, 104). The rate is less than that of conventional rats fed the unautoclaved ration (104). This may explain some of the confusion arising from the Lobund reports since it is difficult to determine whether the "control" animals in that laboratory always were fed the autoclaved ration given the germfree rats.

There is little published information on the growth of the germfree mouse. Gordon (105) stated that germfree male mice (Swiss, albino strain) at 48 to 66 days weighed 28 g whereas conventional animals fed the same autoclaved ration weighed 32 g. For the same age group there was no difference in the weights of the females. These values were for third to eighth generation animals. More recent work indicates that both male and female germfree mice increase in body weight at the same rate as conventional mice of the same strain fed the autoclaved ration (Newton, personal communication). Hand-reared mice at weaning are retarded to about the same extent as rats (62).

The germfree chick has shown a growth response that is as good as, if not better than, that of its conventional control. Forbes and co-workers (59, 106, 107) reported a growth rate of the germfree chick which has approached two and one-half times that of the conventional controls fed the same sterilized diet. It was stated (59) that conventional chicks "grew better on the nonautoclaved diet than on the autoclaved diet." A less dramatic response was seen in turkey poults during the first two weeks, which was the duration of the experiment (58). These reports are in contrast to those from Lobund, where growth of the germfree chick was reported to be considerably less than (108) or equal to (96, 109, 110) that of the conventional controls. This was also reported to be true for turkeys (24).

Comparison of the growth rate of germfree and conventional guinea pigs is complicated by the grossly enlarged ceca in the former. On some rations the ceca are especially large in those animals that grow at rates approaching those of the conventional controls (102). A purified diet similar to that developed by Reid & Briggs (111) produced good growth in germfree guinea pigs and only a slight increase in cecal size (55). Both germfree and conventional guinea pigs that never suckle, either lose weight or do not gain during the first few weeks (42, 49, 55, 63). Thereafter, growth may be at the rate of 1 g per day, with rates approaching 6 g per day after two (55) to four weeks (42).

Only incidental reference has been made to the growth rates of other species. No diet has been developed which permits growth and normal development of rabbits, although some germfree animals have survived for a year (102). A report indicates that a germfree lamb and its conventional control weighed the same at 60 days of age, but thereafter the conventional control gained weight faster (71). A summary of comparative growth rates of germfree animals (including the goat) studied by earlier workers is given by Gustafsson (13) and Reyniers (112).

The poor growth rate of germfree mammals probably is attributable to

the inadequacy of the sterilized diets. They may be deficient in more vitamins than is currently realized. This deficiency may be aggravated when purified diets are in germfree tanks for a week or so. The salts used in some purified rations hasten the rancidity process, which in turn may lead to the destruction of vitamins (113, 114). Sterilization may change the biological value of proteins. Nursing and maternal care may play a more important part in the development of young animals than is commonly recognized. These problems could be studied with conventional mammals that had never suckled their dams.

Antibiotics and growth.—Although most investigators felt that the growth-stimulating action of antibiotics was mediated through the flora in the animal's intestinal tract (115), a final solution to the problem had to await work with germfree animals.

The first report indicated that germfree chicks showed no growth response when various antibiotics were added to the ration (116). Subsequent reports from Lobund (117, 118) stated that low levels of antibiotics added to the rations of germfree birds produced growth stimulation, whereas high levels had no effect. The "low" levels of antibiotics which stimulated growth in the second study were exactly the same (chloromycetin) or two to four times higher (penicillin and terramycin) than those in the first report.

A criticism of the Lobund work is the small number of birds used in any one study and the variety of experiments performed. Two groups (59, 108, 119) rectified these shortcomings and conclusively showed that antibiotics produce no growth stimulation in germfree chicks. In the "clean" environment where these studies were carried out, antibiotics had no growth stimulatory effect in conventional animals. However, when birds were raised in a "contaminated" area, the addition of antibiotics to the ration produced a definite growth response. Even in this case, the birds fed the antibiotic did not grow as fast as the germfree birds (59). Turkey poults also showed no response to dietary antibiotics (58).

Work with germfree chicks has provided a clue as to the mechanism of growth inhibition in conventional chicks raised in "dirty" or "contaminated" quarters. This was shown by Forbes and co-workers (119, 120) to be largely caused by *Clostridium welchii*. When germfree chicks were inoculated with this organism their growth rates were reduced, but could be restored to that of the germfree birds by adding antibiotics to the ration. The antibiotic reduced the number of organisms in the intestinal tract and the ability of these to produce lecithinase (107, 119, 120, 121). Contamination of germfree birds with *Escherichia coli*, *Cl. bifementans*, *Cl. paraputrificum*, *Streptococcus liquefaciens*, or *Lactobacillus lactis* produced no growth depression and antibiotic supplements had no growth effect (107, 120).

Additional evidence that the growth-stimulating action of antibiotics is mediated through the microorganisms in the intestinal tract comes from the observation that the addition of penicillin to a thiamine-low ration, fed to the germfree rat, had no effect on the animal's rate of growth or on the thiamine level of the liver (95). Conventional rats responded to the penicillin with an

increase in growth but with no change in total liver thiamine. Daft (122) indicated that the addition of penicillin or large amounts of ascorbic acid to a pantothenic acid-deficient ration had no ameliorative effect in germfree rats, whereas both supplements prevented the appearance of deficiency symptoms in conventional animals.

Morphological characteristics: general.—Outwardly there is no difference between a healthy germfree animal and its conventional counterpart (123). When the ration is inadequate, germfree animals are likely to present an unkempt appearance, manifest primarily in hair coat or feathers or by a protruding abdomen. The conventional controls may not show these symptoms. The better condition of the controls may be attributable to supplementation of the sterilized ration with fecal nutrients by coprophagy.

Most of the organs and tissues are unaltered in the germfree state. This is true in chicks for the heart, liver, pancreas, gonads, nervous tissue, and thymus (110). There are a few internal differences; some of these appear related directly to the absence of bacteria from the germfree animal (primary differences) while others are more likely associated with present limitations of the germfree technique, e.g., dietary deficiencies (secondary differences).

Morphological characteristics: primary differences.—Glimstedt (39) called attention to the reduced amount of lymphatic tissue with complete absence of secondary nodules in the germfree guinea pig (124). The reduction was pronounced in those organs normally in contact with bacteria. This observation has been confirmed and extended to the nasal cavity of guinea pigs (49). Monocontamination of the germfree guinea pig changes its lymphatic system to that of the conventional animal (49). Germfree chicks at two weeks of age and weanling germfree rats also show reduced lymphatic development (125). Neither plasma cells nor secondary nodules were seen in the lymph nodes of the ceca or other intestinal areas of germfree chicks. Germfree rats, monocontaminated for one to two weeks, showed newly developing secondary nodules in the mesenteric lymph nodes and immature plasma cells in the spleen. It has been reported that growth stimulation produced in conventional animals by antibiotics is associated with a change in the lymphatic system of the intestinal tract to resemble that in the germfree state (126).

Nuttall & Thierfelder (31) noticed the marked enlargement of the ceca in germfree guinea pigs. Similar observations have been made by all subsequent investigators—the cecum sometimes constituting as much as half the body weight (112). In some cases, enlargement of the cecum was not attributable to an increase in weight of cecal tissue (127).

The cecal contents from the conventional guinea pig contained 1.3 times as much dry matter as from the germfree animal (127). There was an increase in the concentration of nitrogen, ash, and crude fiber with a marked decrease in total lipids in the germfree cecal contents when a correction was made for the difference in dry matter. Studies such as this should be extended.

The germfree rat may reproduce even though its cecum is five times larger than that of the conventional animal (128). The young from such mothers, when raised through the weaning period on their mothers' milk, showed cecal

enlargement by the second week. By the 25th day of life, their ceca were five to seven times larger than those of conventional rats. This difference continued into adulthood. Further study showed that both the cecal contents and, to a lesser extent, the cecum itself were enlarged (26, 67, 105, 129). Although most germfree rats show enlarged ceca, Zweifach and co-workers (129) reported that two of their germfree rats had small ceca and that one conventional rat, fed the same purified-type ration, had an enlarged cecum. Unfortunately, these workers did not present a more complete report on their animals.

The cecal contents of germfree rats had two-thirds as much dry matter as had the cecal contents of conventional animals (128). Neither the increased moisture nor the amount of cecal contents was lowered by a drastic reduction in dietary salt (128). The concentration of histamine in the cecal contents of the germfree rats was one-fifth that in the conventionals. The later work was possibly prompted by Gordon's suggestion (105) that the large cecum was probably a result of poor muscle tone. A barium meal passes through the intestinal tract of the germfree rat as rapidly as through that of the conventional animal, in spite of the difference in cecal size (130).

Bacterial contamination produces a reduction in the size of the cecum. Within five to seven hours after the germfree animal is brought into the animal room, there is some reduction in cecal volume (105, 131) but it requires four to seven days for the cecum to reach its "normal" size (67, 105). Reduction in cecal size is not due to the presence of bacteria per se since monocontamination with *Proteus vulgaris*, *E. coli*, or *L. acidophilus* produced no change even after eight days (67). The reduction in cecal size is associated with a change in the color of its contents from greenish-brown to grey (67).

Mice in the germfree state are similar to rats as far as the cecum is concerned, but much less work has been done with these animals (105). In some strains of germfree mice, the ceca become so large that they twist, forming a volvulus which results in death (132). Cecal size in germfree chicks is the same as that of conventional birds (133).

The enlarged ceca in germfree mammals poses a number of difficulties and some interesting problems for future research. A possible deterrent to reproduction and rearing of germfree guinea pigs is their enlarged cecum. When these animals produced living young, the dam usually died with the cecal wall protruding through either the anus or vagina (Horton & McDaniel, personal communication). The "true" weights of germfree guinea pigs and rabbits cannot be secured prior to autopsy because of the enlarged ceca. If the growth rate of the germfree guinea pig is kept to a minimum, the cecum does not become as large as if the animal is permitted to eat *ad libitum* (102).

Dietary factors should probably be considered in any attempt to control cecal size in germfree animals. Germfree rats (103) and guinea pigs (55) fed a practical type ration had larger ceca than those fed a purified type ration. The carbohydrate also may have some influence, since, with conventional

rats, a ration containing lactose produced ceca that were three times as heavy as those in animals receiving glucose (134).

The oral cavity of the germfree chick was similar to that of conventional birds, while the mucosa of the esophagus and crop of the germfree chicks was smoother (133). The proventriculus in germfree chicks was lighter in weight than that of conventional birds, with the difference increasing from 10 per cent in the two-week birds to 40 per cent in birds of six months or more. The small intestine appeared paler, slightly shorter in length, and definitely lighter in weight for the germfree chicks. The latter was probably the result of dehydration, since the dry weight of the small intestine was greater in germfree chicks (133). The intestine of the germfree chick contained less connective tissue than that of the conventional bird (105). The intestinal tract of germfree guinea pigs down to the cecum is "normal" (63).

There was no difference in the clotting time of blood from germfree and conventional chicks, but in younger animals both the number of red cells and the hemoglobin level were slightly higher in the germfree chicks (133). The differences in both red cells and hemoglobin disappeared in older birds. Germfree guinea pigs have the same values for hemoglobin and hematocrit as conventional animals but a lower leucocyte count (55). The white cells, especially lymphocytes and monocytes, were present in much lower numbers in germfree birds; the numbers of heterophils, acidophils, and basophils were similar in both groups (133). A similar situation exists in the germfree guinea pig (63). The statement has been made that the germfree rat differs from the above but no data are available (26).

The low white counts aid in explaining the slower rate at which *E. coli* injected into the blood are cleared by germfree mice (135). Other factors are also operative, since *Staphylococcus aureus* was cleared from the blood at the same rate by germfree and conventional mice.

Data are not available on the weight of the total skeleton in germfree animals but it is stated that the "germfree subjects" have a heavier skeleton (26). Chick bones show no differences in proximate and vitamin composition for the germfree and conventional animals (133).

After two months of age, the lungs of germfree chicks are lighter in relation to their body weight than are the lungs of conventional birds (133). This is probably of no significance because of the overlapping of values for the two groups. Lung consolidation in mice (132) and the same with hyperemia in rabbits (66) have been reported but these changes are probably not the result of the germfree state.

Morphological characteristics: secondary differences.—A variety of changes observed in germfree animals are probably related to some environmental factors, or factors that must be overcome before the animals can be considered "normal." The absence of "signs of recent ovulation" in the guinea pig (63) must have disappeared in animals fed a different diet since there have been a number of pregnant germfree animals (55, 68). Enlargement of the adrenals in germfree rats disappeared after more space was provided

(103). The loss of hair in the weanling hand-raised germfree rabbits (66) will probably disappear as soon as the young can be suckled. The cataracts in weanling rats disappeared when the young were nursed by their mothers (23). Feces from germfree chicks are slightly fluid (133). The same is true for rats even after a number of generations (Daft & McDaniel, personal communication). It may be that the latter, together with the absence of odor (133), may be a primary characteristic of the germfree state.

Reproduction.—The fact that at least 14 generations of rats and 11 of mice have been reared (47) proves that repeated reproduction is possible in some species of germfree animals. It is stated that "the germfree animal matures and begins its cycle of reproduction at the same age as, or a little earlier than the normal conventionals" (123). Germfree rats produce and wean as many young per litter as do conventional animals fed the same sterilized ration (46, 136). All laboratories have not been so successful in the reproductive performance of their germfree rats: György (107) states that after one or two litters the rats become sterile.

Germfree hens produced eggs at the same age as conventional birds (133) and when three of these fertilized eggs were incubated, one chick hatched (61).

Germfree guinea pigs possessed viable sperm. A number of pregnant animals have been secured and some young born but the enlarged ceca interfered with rearing of the young and subsequent welfare of the dam (55, 68).

Until recently, there were no reports of pregnancies in germfree rabbits even though these animals have been maintained beyond the breeding age (66). A contaminated female, removed from the germfree tank, bore and reared a litter after mating with a conventional male (66), suggesting that the ovaries were functional. Second generation germfree rabbits have been obtained, but "no young born from germfree mothers has as yet survived" (66a).

Life span.—Lobund is the only laboratory which has reported on the life span of germfree animals. Germfree rats and mice live to practically the same age as conventional animals (47, 102, 132, 137). Germfree chickens, monkeys, and guinea pigs have been maintained for 400 days (47, 63).

Causes of death.—Again, the little information available comes from Lobund. The experience at that laboratory appears to have changed over the years. Originally the large ceca in the germfree rats frequently twisted to form a volvulus which led to death (Hawk, personal communication). More recently the ceca in the rats appear to have become smaller. From 1954 to 1956, volvulus caused one-fourth of the deaths (138), asphyxia was responsible for half, and other lung disturbances were responsible for most of the others in germfree rats.

In the guinea pig, volvulus of the enlarged cecum is the primary cause of death (63). Poor nutrition is another major factor in the early mortality of the guinea pigs [Daft & McDaniel, personal communication (63)].

Female germfree C_3H mice showed a higher mortality rate than mal's

(39 vs 6 per cent) (132). Half the females that died had extensive deposits of calcium oxalate in the bladder and urethra; the kidneys, heart, and lungs also showed extensive calcification. Male germfree mice and conventional animals showed no such changes. Twisted ceca were responsible for the death of about one-eighth of the females and most of the males. The oxalate deposits in these mice should be studied to see if a pyridoxine deficiency is related thereto (139).

Germfree Swiss mice do not show any calcification. In this strain, the primary cause of death is asphyxiation, with other lung disturbances accounting for most of the rest (138). Some of the lung disturbances may have been caused by viral infections but attempts to isolate viruses from the tissues have been unsuccessful.

BIOCHEMICAL STUDIES

Composition of tissues and organs.—The concentration of ascorbic acid and total cholesterol in the adrenal glands is the same in germfree and conventional chicks (105).

The blood of germfree animals has low levels of certain serum proteins. The concentration of total serum proteins remains unchanged by the germfree state in chicks and is reduced by 25 per cent in guinea pigs.

The 35-day-old germfree chick has a γ -globulin concentration equal to 75 per cent of that of the conventional bird. While the level increases three-fold in the conventional bird up to 125 days of age, it shows no change in the germfree bird. The albumin level is slightly higher in the germfree chick; consequently, there is practically no difference in total protein levels (109, 110, 140, 141).

Not all bacteria increase the γ -globulin level in germfree chicks. This was shown by the accidental contamination of four-week-old chicks with an unidentified bacterium (110). Contamination of newly hatched chicks with *Clostridium perfringens* increased the γ -globulin level but did not bring it up to that of conventional birds (142). In the latter case, the birds had been contaminated for one month. Contamination under similar circumstances with *Streptococcus faecalis* restored the γ -globulin level to that of conventional birds (142). The germfree animal provides an excellent tool for studying the component of bacterial cells which stimulates the synthesis of γ -globulin.

The titers of complement, properidin, and of some heterohemagglutinins are the same in germfree and conventional birds. Conventional birds develop immune bodies against the common intestinal bacteria early in life; the blood of germfree birds contains antibodies only for those bacteria which are present in the sterilized diet in a nonviable form (87). Germfree chicks were used to show that "anti-human blood group B agglutinins in White Leghorn chicks are acquired early in life and are not inherited" thus settling a problem that has plagued immunologists for over 30 years (143).

In the germfree rat the serum globulin fractions are all lower than in the conventional animal with the level of the γ fraction about 30 per cent of that

of the conventional rat. There was no change in the γ -globulin levels for successive generations of rats born to and reared by germfree parents (72). Here, as in the chick, the albumin level was slightly increased, with no significant difference in total serum protein levels (72, 140, 144).

When the germfree rat became contaminated, the levels of β - and α -globulin fractions increased during the subsequent two weeks, whereas the γ -globulin fraction started to increase only thereafter and did not attain its maximum level for one to two months (131, 140, 144, 145, 145a). The latter finding is in marked contrast to the appearance of antibodies within two days after rats are immunized with sheep red cells or typhoid H-antigen. In these cases, maximum antibody production was attained by the seventh day (146, 147). In contrast to the rat, the germfree chick produces hetero-hemagglutinins or antibodies at a rate and to a titer that equals conventional birds (148).

Germfree guinea pigs differ in that the level of total serum protein was only 75 per cent of that of the conventional animals; a reduction was apparent in all proteins studied. Beginning with the third week of life, the germfree guinea pigs had lower levels of albumin and α -globulin than conventional animals fed the sterilized diet. The levels of γ -globulin in both the germfree and conventional guinea pigs fed the sterilized diet were equal at twelve weeks of age; they were, however, lower than the levels in the conventional animals fed the unsterilized diet (64). Diet appears to influence the presence of γ -globulin in the germfree guinea pig, since the animals had no γ -globulin when fed a sterilized laboratory Chow [(145a)]. The serum complement level in germfree guinea pigs increases with age at the same rate as in conventional animals, thus showing that living microorganisms are not essential for its synthesis (149).

A germfree lamb, four months of age, had 4.6 g of protein per 100 ml of serum, while the conventional lamb had 5.7 (71).

There is no difference in hemoglobin levels of germfree and conventional chicks throughout their life span (133). A similar statement has been made for the guinea pig (42, 55). Germfree rats (90) and mice (132) have slightly lower levels than conventional animals. A germfree lamb had 9.0 g of hemoglobin per 100 ml of blood, while the conventional animal had 12.4 g (71).

There was no difference in serum cholesterol levels of six- and eight-week-old germfree and conventional chicks fed a cornstarch-casein diet (106). However, dietary carbohydrate influences the cholesterol level in germfree as well as in conventional chicks. The addition of cholesterol to a purified sucrose ration produced higher serum cholesterol levels than when glucose was used. The germfree chicks had higher serum cholesterol levels than the conventional animals fed the same sterilized ration when it contained glucose but not sucrose (150). The germfree rat, however, has a serum cholesterol level that is 1.5 times that of the conventional animal fed the same sterol-free sterilized ration. When 0.5 per cent cholesterol was added to the ration, the serum cholesterol levels increased in both groups to essenti-

ally the same value (151). Feces from germfree rats that were fed the basal ration showed the presence of only cholesterol, while the feces from conventional animals showed a number of neutral sterols, with coprostanol the major one (151).

The ammonia level in the portal blood of the germfree guinea pig is considerably lower than that of the conventional control. The values increased for both groups as the protein in the ration was raised from 0 to 40 per cent, with the conventional animals showing the greater rate of increase. The ammonia levels in the peripheral blood were from one-half to one-fourth those of the portal blood, providing evidence for the removal of ammonia by the liver in both groups of animals (74). The much higher levels of ammonia in the portal blood of conventional animals confirm the previously unproved hypothesis that most of the ammonia in the portal blood is a product of bacterial action in the intestinal tract. Confirmation of this comes from the work wherein labeled urea was injected into germfree and conventional rats. The latter expired large amounts of labeled CO_2 whereas the germfree rats expired very little (75). The labeled CO_2 expired by the germfree rats came presumably from nonenzymatic hydrolysis of urea. Whether this explanation can be applied to the blood ammonia in the germfree guinea pig remains to be tested.

The composition of bone from germfree and conventional chicks is reported to be similar (133). An exception to this is fluoride, which in germfree rats is 75 per cent of that in conventional animals fed the same purified ration (23). Whether this difference has any biological significance is an open question.

The feces of most germfree animals appear to be softer and more fluid than those of conventional rats. It has been stated that "the feces of the germfree rats may be higher in moisture (25 to 30 per cent) than those of conventional rats (20 to 25 per cent), the fecal nitrogen is possibly higher in germfree rats while the total quantity of fat excreted was very similar" (90). The same report states that both groups of rats consumed the same amount of water. It is possible that the fecal loss of nitrogen by the germfree animal is higher than that of the conventional, since a similar report comes from the Walter Reed laboratory (Levenson, personal communication). The higher fecal loss of nitrogen by germfree animals is intriguing, since it is generally assumed that about half the nitrogen in the feces of conventional rats is derived from intestinal bacteria. These observations raise a number of questions: Is the increased nitrogen in the feces of the germfree animal associated with the increased size of the cecum or with the increased content of fecal tryptic enzymes (152)? If the fecal nitrogen in the germfree animal represents sloughed mucosa, does this animal have a more rapid turnover of mucosal tissue? Or, does the difference in fecal nitrogen represent fundamental differences in protein metabolism resulting in a higher loss of nitrogen into the intestine of the germfree rat? It is unfortunate that so few studies have been made on the feces of germfree animals—nothing has been done to characterize the fecal nitrogen.

Germfree rats excrete in their feces 1 to 6 mg of trypsin and 12 to 25 units of invertase per day, while feces from conventional animals contain neither enzyme. The fecal amylase content of germfree rats was the same or slightly higher than for conventional rats. When germfree rats were contaminated with feces from conventional rats their fecal enzymes returned to "normal" levels within 24 hours (152). Does the trypsin have any effect on the gastrointestinal tract of the germfree rat? It would be important to know whether trypsin occurs in the cecum of the germfree guinea pig and, if so, is this responsible for its enlargement? The intestinal contents of germfree rats had the same concentration of maltase, invertase, and oligo-1,6-glucosidase activity as in conventional rats (153).

It is unfortunate that there are no recent data on the composition of urine from germfree animals. No indican-like compounds were found in the urine of germfree rats, whereas there was a "high indicane content" in the urine of germfree rats contaminated with *E. coli* (67). There was no sterco-bilin (tetra-hydromesobilin-*b*) or urobilin (mesobilin-*b*) in the urine of germfree rats, while "conventional and ex-germfree rats showed normal values" (67).

Livers of germfree chicks contained the same amount of moisture, ash, nitrogen, phosphorus, thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, folic acid, and vitamin B₁₂, but the ascorbic acid content was only one-fourth that of conventional birds (96). The low ascorbic acid level in the liver was in contrast to the "normal" values in muscle, brain, and adrenals.

Dietary liver necrosis does not develop in germfree rats fed *ad libitum* a ration low in vitamin E. However, when their food intake is restricted to that of conventional rats, both groups develop liver necrosis (154). This is an intriguing observation and appears difficult to explain. If this observation is confirmed it poses an enigma to nutritional dogma which holds that a deficiency is less likely to develop in a slower-growing animal.

Liver cirrhosis, in contrast to dietary liver necrosis, develops more rapidly in germfree rats fed a choline-deficient diet (155). This suggests that, in conventional animals, certain bacteria produce an "anti-cirrhotic nutrient" in amounts just sufficient to postpone the development of cirrhosis. The "nutrient" would not be available to germfree rats.

Germfree rats fed a protein-deficient cirrhogenic or pantothenic acid-deficient ration show white stipples scattered irregularly throughout the lungs; they measure up to 1.5³ mm in diameter and consist of xanthoma cells containing lipid (156).

Metabolism.—Labeled bile acids fed to conventional rats treated with antibiotics had a half life of six to seven days compared to 2.5 days in the controls (157). In similar experiments performed with germfree rats, the half life was from seven to eleven days. Conventional rats excrete a variety of bile salts, whereas the germfree and antibiotic-treated conventional rats excreted only taurocholic acid. These observations indicate that the bacteria and not the intestinal enzymes are responsible for the conversion of the bile sterols. The size of the bile-acid pool in germfree rats was 15.4 g per 100 g

body weight (130) compared to 6.0 in conventional animals (158). Contamination of the germfree rat with *Aspergillus niger* had no effect on either the half life or fate of labeled cholic acid. Additional contamination with *Cl. perfringens* also had no effect on the half life but resulted in the conversion of the bile acid to a variety of compounds (130, 159).

Histamine is thought to be produced by bacterial decarboxylation of histidine in the intestine, with a small amount formed endogenously. The fact that bacterial action plays an insignificant role in this process was shown by the similarity in the urinary histamine excretion of germfree and conventional rats (160).

Methyl synthesis by germfree animals and its incorporation into tissue choline and creatine at rates comparable to those of conventional rats have been known for some time (161, 162). As a supplement to these studies, it was shown that the conversion of choline to trimethylamine is mediated through the bacteria in the intestine. Only 0.6 per cent of the administered choline appeared in the urine of germfree rats as trimethylamine, whereas 37 per cent appeared in conventional rats (163). The latter study resolved the conflict as to whether bacteria (164) or the liver (165, 166) played the major role in the breakdown of choline to trimethylamine.

Shock.—There is considerable circumstantial evidence to support the thesis that "irreversible" shock results from endotoxins released by bacteria in the intestinal tract (88, 129, 167). For this reason, it is surprising to find that germfree rats subjected to severe bleeding went into irreversible shock as frequently as conventional animals fed the same ration. The gross pathological changes were similar in both groups (167). The same was true of germfree mice subjected to tourniquet shock, while germfree chicks subjected to this treatment showed an increased susceptibility to shock (Levenson, personal communication).

Starvation.—The complete absence of food produces earlier death in germfree mice than in conventional animals or in those monocontaminated with *E. coli* (Levenson, personal communication). Attempts are now under way to determine the cause of this unexpected behavior.

Wound healing.—Wound healing in the germfree guinea pig shows some histological differences from that in the conventional animal (14). Transplants of skin from one germfree guinea pig to another remained viable through the 15th day of the study, while those in conventional animals had undergone complete necrosis by the 6th day.

x-Irradiation.—x-Irradiation of germfree rats with doses above 400 r resulted in survival times that were twice as long as those of conventional animals (168). A similar situation exists for germfree mice (169). This is not too surprising, since many of the secondary effects of irradiation injury are traceable to invasion of the tissues by bacteria normally confined to the intestinal tract (170, 171, 172). The preceding agrees with a report that germfree chicks show longer survival times than conventional birds for both high and low doses of x-irradiation, but the ultimate percentage survival was the same for both groups (173).

MICROBIOLOGICAL STUDIES

Sensitivity of germfree animals to infection.—Some microorganisms cannot be established in germfree animals; others show a predilection for germfree animals while still others behave the same in both. Phillips and co-workers were the first to show a differential response of the germfree animal to infection with *Entamoeba histolytica* (174). To cultivate this parasite *in vitro* requires the presence of another organism such as *Trypanosoma cruzi*. When a suspension of these two organisms was injected into the ceca of germfree guinea pigs, no ulcerative lesions developed and no amebae were observed in animals sacrificed after the fifth day. In the conventional animals, 34 out of 37 showed acute ulcerative amebiasis with the other three showing amebae on the 21st day after inoculation. Even though *E. histolytica* did not become established in the germfree guinea pig, the organism accompanying it, *T. cruzi*, was found in 12 of 17 germfree guinea pigs; the blood of all conventional animals was negative for this organism. Monocontamination of the germfree guinea pig with either *E. coli* or *Aerobacter aerogenes*, prior to inoculation with the *E. histolytica* preparation, produced a response similar to that of the conventional animals.

The resistance of the ceca of germfree guinea pigs to invasion by *E. histolytica* is all the more interesting in view of the enlargement and the consequent thinning of the wall of this organ. The higher oxidation-reduction potential of the cecal contents from germfree guinea pigs prompted the addition of sodium thioglycolate or L-cysteine-HCl to the inoculating media. When this was done, small ulcerative lesions appeared on the cecal wall surrounding the inoculation site (76, 175). The limitation of the ulcer to the inoculation site may be a reflection of the inability of the reducing agents to influence anything more than a small area within the cecum, or it may be associated with the puncture made at the time of inoculation. Evidence for the latter comes from the observation that when the cecal wall was traumatized, lesions occurred at those sites following inoculation. To determine whether the inoculum used in the germfree tank was still potent, germfree guinea pigs were removed from the tank 24 hours after inoculation with the *E. histolytica* preparation. All animals so treated showed severe ulcerations.

The above observations suggest that *E. histolytica* requires something for its growth which is produced by the bacteria in the intestinal tract. It was possible only recently to produce *in vitro* a pure culture of this organism which could be subcultured (176).

Although not directly related to infection, it is interesting to note that germfree guinea pigs are resistant to the intraperitoneal injection of sterile agar, whereas conventional animals so treated died with severe symptoms (14).

In contrast, there are a number of organisms which appear more virulent in germfree animals than in conventionals. One of these is *Trichomonas vaginalis*. When injected subcutaneously into germfree guinea pigs it produced "tissue erosions, numerous active organisms, considerable pus or

caseous material, and often relatively large amounts of gas. . . ." Infection was established more easily in germfree animals, whereas it occurred less frequently in conventional guinea pigs and then only as a small cyst (73, 80). Another organism that behaves in a similar manner is *Bacillus subtilis*. It is highly pathogenic to germfree guinea pigs, whereas conventional animals are not affected by it (177).

There are statements that germfree rats and mice can be infected with *Shigella dysenteriae* but no symptoms or ulcerative lesions are produced, whereas conventional animals will not harbor the organism after inoculation (178). Germfree guinea pigs are even more susceptible in that they die within a few days after being challenged with *S. flexneri*, while conventional animals do not even harbor the organism in their gastrointestinal tracts. Monocontamination of germfree guinea pigs with *E. coli* protected them against *S. flexneri*; this was not true of monocontamination with a lactobacillus isolated from a conventional guinea pig (179). Since *E. coli* has been reported to be present irregularly in the gastrointestinal tracts of normal guinea pigs, there must be other organisms which protect the conventional guinea pig from *Shigella* infection.

A number of parasites which normally infect rats and mice become established when inoculated into germfree guinea pigs but not when inoculated into conventional guinea pigs. These include the rat nematode *Nippostrongylus muris*, the mouse nematode *Nematospiroides dubius*, and the mouse tapeworm *Hymenolepis nana*. All three were able to grow to maturity and produce eggs in the germfree guinea pigs (180).

Even though some germfree animals are more susceptible to infection than conventional animals, this does not mean that all strains of a given species are. This is suggested by the statement (178) that a hepatitis virus which produced no effect in conventional birds produced a "fatal disease" in germfree White Leghorn chicks. Germfree New Hampshire Reds, however, were resistant to the infection.

Finally, for *Ascaris lumbricoides* there was no difference in the infectivity of germfree and conventional guinea pigs to eggs of this worm (80).

Immune response.—Although the level of γ -globulin in the serum of germfree animals is much lower than that of conventional animals, they respond to challenges with bacteria by producing globulin. This was evident when germfree rats were infected (via stomach and colon) with a suspension of feces from conventional animals. The serum globulin fractions began to increase one week after exposure, and had almost reached the levels of conventional rats by the fourth week (144). Similar evidence has been presented for germfree chickens monocontaminated with *Cl. perfringens* or *S. faecalis* (181).

Exposure of germfree animals to contaminated areas.—When germfree rats (85 to 98 days old) were removed from their sterile environment, they were given orally, as well as rectally, a suspension of cecal contents from conventional rats. This heavy infection had little effect on the survival of the animals (131).

Germfree chicks are equally resistant to such exposure, since they can be put directly into a barnyard without any apparent harm (Forbes, personal communication). Guinea pigs do not survive removal from the germfree environment. However, an occasional animal has survived after having been previously contaminated with a single bacterial species prior to removal from the unit (Newton, personal communication). Germfree C₃H mice, especially those under one month of age, show a high mortality when removed from the sterile environment. Germfree Swiss mice are more resistant, with 80 per cent surviving exposure to the animal quarters (132). Again, it is unfortunate that so little factual or even precise descriptive information is available on the behavior of the germfree animals during the transition from the germfree to the conventional state.

Viral sensitivity.—A viral disease was reported to exist among the germfree chicks at Lobund, but was not seen among the conventional birds hatched from the same clutch of eggs. The severe body tremor and unsteady gait were reportedly attributable to cerebellar and medullary damage. Monocontamination of germfree chicks from birth with either an unidentified Gram-negative rod or *S. faecalis* did not prevent the development of the disease (18). Since no virus was isolated from the affected chicks, it is possible that a nutritional deficiency caused the disease. There is a variety of dietary deficiencies which produce symptoms similar to those seen in these chicks.

An analogous situation has been reported for germfree guinea pigs. A highly fatal bronchopneumonia-like condition was the most likely cause of death of the 10- to 14-day-old animals. Conventional guinea pigs remained healthy when permitted to suckle their dams, whereas a few conventional animals which were not so privileged developed symptoms of the disease. Attempts to prevent the disease by dietary and chemotherapeutic means were unsuccessful. Feeding the germfree guinea pigs an autoclaved suspension of cecal contents from conventional guinea pigs prevented the disease. When a new source of guinea pigs was secured, the disease disappeared (182).

Etiology of disease.—The role of various organisms in a chronic respiratory disease, "air sac" infection, in chickens and turkeys has been controversial. Inoculation of germfree chicks and turkey poults with a culture of *Mycoplasma gallinarum* free from other bacteria produced the "air sac" infection and definitely showed that this was the only organism involved in the etiology of the condition (183).

DENTAL STUDIES

Irrefutable proof that dental caries result from bacterial action came from studies on germfree animals. When weanling rats, both conventional and germfree, were fed a casein-rice type of cariogenic diet with a 5 per cent sucrose solution as the source of water, the conventional rats developed severe and extensive caries. The 22 germfree rats, however, showed no signs of caries even when sections of the teeth were examined microscopically. In the germfree group, there were four molars "where a cusp or some other

coronal aspect had been definitely fractured, probably on a traumatic basis, yet showing no evidence of caries." The teeth in the germfree rats were normal morphologically and showed the usual occlusal wear of the cuspal areas, but the mouths of these animals were much cleaner than those of conventional rats. The scarcity of stains on the teeth of the germfree animals was another reflection of the absence of bacteria (23, 184).

As an extension of this work, a strain of *S. faecalis* and an alcaligenes-like bacterium isolated from carious molar teeth of conventional rats were used to swab the mouths of germfree weanling rats. Again, the germfree rats which had not been monocontaminated remained caries-free. All those that were inoculated with *S. faecalis* developed severe caries. The presence of the proteolytic organism (the alcaligenes-like bacterium) had no influence on caries development (77). On the basis of these observations, it is obvious that dental caries will develop only in the presence of specific bacteria and that these bacteria are probably acid producers.

Additional work showed that an unidentified microaerophilic " *α -hemolytic Streptococcus*" will also produce typical caries in germfree rats within a period of two months (104). The teeth of the germfree animals remained free of caries in spite of considerable food impaction and occasional fractures of the molar cusp.

Germfree mice showed alveolar bone changes comparable in onset and scope to those seen in conventional animals with periodontal disease. They also showed calculus-like deposits on the enamel surfaces of the molar teeth (185). The dental calculus deposits in germfree rats resembled those seen in conventional animals on the basis of staining reactions and x-ray diffraction patterns (186).

TUMOR STUDIES

Germfree animals will respond to induced tumor development. This was shown when the Yoshida sarcoma cells of rats were injected into the abdominal cavity of the germfree guinea pig. There the sarcoma cells grew rapidly and showed mitotic activity (14). The fibrosarcoma that develops in chicks after an injection of methylcholanthrene is not transmissible when removed from conventional birds. It is transmissible when produced by the same technique in germfree chicks (187).

Conventional C₃H mice show a high incidence of spontaneous mammary tumors in females and a high incidence of hepatomas in males. At the time of the report, there had been no tumors in germfree mice of this strain (132). The mammary tumor agent does not pass the placental barrier, which explains the low incidence of these tumors in the germfree C₃H mice (they were originally secured by Caesarian section and never nursed their mothers). It would be important to know whether these germfree mice develop mammary tumors when bred intensively, since conventional C₃H mice that were suckled only by foster dams from resistant strains, when so bred, developed a high incidence of mammary tumors (188, 189). Swiss mice normally show a

low incidence of spontaneous tumors. This presumably is not changed by the germfree state. Germfree mice, however, develop tumors when challenged with methylcholanthrene or dietary stress and those tumors can be transmitted, as in the germfree chick (190).

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BIOCHEMISTRY OF CANCER¹

(IMMUNOLOGICAL ASPECTS)

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"Cancer is in principle a curable malady," concluded Gaylord in his report to the Commissioner of Health of the State of New York (1). Dr. Gaylord had taken for the basis of his optimism a series of preliminary immunity experiments which he & Clowes, along with their assistant, Baeslack, had carried out during the latter months of 1904 (2, 3), and a confirmatory series which Clowes & Baeslack had performed during the first six months of 1905 (4). In the completed series it had been shown that the serums of mice which had "spontaneously recovered" from the inoculations of a Jensen mouse adenocarcinoma contained immune bodies which destroyed the transplantability of Jensen tumor inoculums when mixed with the serums. These same mice had also been found to be resistant to reinoculations of the Jensen tumor.

It seems particularly fitting that this special report for *Annual Reviews* on the current immunological climate of cancer research should stem from the same laboratories in which the first cancer immunity experiments were performed; however, in this same corner from which Gaylord viewed the prospects of immunological control of cancer so optimistically, this reviewer does not yet find the rôle of cancer immunology to be one of any real immediate value to the cancer clinician. This is not to say that 58 years of research in this field have been for naught, for probably no other discipline has been more successfully applied to the cancer problem in probing its mysterious nature than immunology. It would not be too far from the truth to say that in every current study, from carcinogenesis to chemotherapy, involvement of an immunological mechanism must at least in part be suspect.

CANCER ANTIGENS

The question uppermost in the cancer immunologist's mind is whether tumors contain substances exclusive of normal tissues, and whether such substances can be exploited in immunotherapy. Milgrom (5), for example, qualified the optimistic conclusion of his review with the statement that the final outcome of effective immunization would depend upon the question of whether tumors really possessed cancer-specific antigens.

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Cytolipins.—Since the days when Hirszfeld and his co-workers (6) and Witebsky (7) began the serological search for specificity, the complement-fixation reaction (CFR) has been a favored technique, and a version of it was developed by Rapport & Graf (8) to investigate cancer lipids. They found it very useful in the detection of cytolipin H (9) preceding isolation and characterization (10, 11). Graf & Rapport (12) then found that out of 17 anti-human-cancer antisera, 14 reacted strongly with cytolipin H, but all reacted poorly with extracts from normal tissues. Through the use of anti-cancer antisera that reacted with extracts of gastrointestinal tract tumors but not with cytolipin H or other known lipids, Graf, Rapport & Brandt (13) looked for other lipids and found, after employing silicic-acid chromatography, a new one called cytolipin G. To date, at least six independent lipid systems have been demonstrated in this way through the use of antihuman-tumor antisera (14).

Cytolipin H was found to have the structure of a ceramide lactoside consisting of four units—fatty acid, lipid base, glucose, and galactose. The lactoside structure was confirmed immunochemically by showing that an antibody prepared against a protein-azophenyl-lactose antigen would cross-react with cytolipin H (10, 11, 14). A synthetic cytolipin, similar to H, has now been prepared (15). The discoverers of malignolipin wrote that their phospholipid, extracted from human tumors, could be found "only in malignant and never in normal tissues" (16). Rapport and co-workers did not hold cytolipin H in such high regard and were constantly on the lookout for sources in nonmalignant tissues. How they happened to choose ox spleen as a possible source and isolated from it a lipid immunochemically identical to cytolipin H was as pleasurable reading to this reviewer as any in modern forensic fiction (17).

Agar-gel diffusion of antigens.—Korngold (18) and Zilber (19, 20, 21) have reviewed the subject of cancer-specific antigens from the standpoint of two other methods: agar-gel double diffusion and sensitization-desensitization-anaphylaxis reactions. Korngold's group lately has been particularly involved in comparing normal and leukemic leukocyte antigens (22, 23), and have been able to identify several: one is specific for granulocytes, another for leukocytes from acute-leukemia patients, but none is specific for lymphocytes. Antigenic differences in granulocytes between patients with chronic myelogenous leukemia and those without were not found. Messineo (24), using the agar-gel diffusion technique, has recently reported an immunological difference between the deoxyribonucleoproteins of normal- and leukemic-human white cells. Miller (25) has described distinct precipitin lines in agar gel for three antigens from an alcoholic extract of rat sarcoma, two of which also formed from an extract of a rat lymphoma but none from extracts of muscle, spleen, kidney, lung, or plasma. Diadkova (26) has used agar-gel columns effectively in showing a community of similar antigens in three chick sarcomas as well as antigens distinct for each tumor.

Cancer-specific antigens and the sensitization-desensitization-anaphylaxis

method.—Many of the papers referred to by Zilber (20) were given at a symposium in Moscow in 1956 and briefly reviewed by Grabar (27) in 1957; the English edition (28) did not appear until 1959. Several of the chapters illustrate the method of sensitization-desensitization-anaphylaxis by which cancer-specific antigens may be detected. One paper (29) is of particular interest. A fibroadenoma and a sarcoma had developed simultaneously in the same rat. An extract of each was used to sensitize guinea pigs, and extracts of normal rat tissues and of the tumor opposite to the original challenge were used to desensitize the pigs. When tested for anaphylaxis with the original tumor extract, the guinea pigs still reacted. Thus, each of the two malignancies in the same rat was shown to have its own individual substances as well as some in common with the other tumor and with normal tissues. Although nucleoproteins from the so-called Zilber fraction have been thought to be mainly responsible for the specificities involved, Mazina (30) describes a "full" antigen of human cancer tissue in which activity, as shown by sensitization-desensitization-anaphylaxis experiments, resides in the lipid and polysaccharide fractions but not in the protein fraction of the hydrolyzed antigen. The "full" antigen is described, moreover, as 10 to 90 times more active in complement fixation reactions than aqueous saline extracts.

Antigens of nonmalignant diseases.—Boeva (31, 32) has extracted substances from preneoplastic lesions of the human mammary gland and, by use of the sensitization-desensitization-anaphylaxis method, has found evidence in 42 cases of antigens different from those in normal mammary gland extracts. Since chronic mastitis is not a condition necessarily leading to malignancy, the production of substances in mammary gland tissue inflicted with mastitis that are different from the substances in normal mammary tissue would not appear to denote the presence of malignancy but only of some pathological condition. The same may be said for the "spleen autoantigens" in leukoses (33) which are brought out possibly because of nonspecific inflammatory and degenerative processes rather than specifically by tumor. The "necrotic antigens" of Hirszfeld (34) and of Milgrom (35) also come to mind—substances obtained from tuberculous tissues and exudates which react with the same specific antisera as antigens from malignant tissues. Milgrom (5) was certainly right to caution us that to contrast malignant tumor antigens with normal tissue antigens is not enough to establish true specificity. The contrast must also be made with tissues from subjects with nonmalignant abnormalities.

Antigens vs. absorbed serums.—The antigens which Björklund's group (36) prepared from 60 human carcinomas were insoluble, heat-labile substances obtained by ether extraction. Details of the extraction procedures have been reported earlier (37). The test which the group used was based on the cytotoxic action of antisera upon cultured human cancer cells in the presence of complement. Specificity of the reaction for detecting unique cancer substances depended upon the absorption of their antisera with normal tissue preparations. In the work reported above such absorptions were mentioned

as having been carried out at an earlier time, but the reference was incorrectly given (37) and should have been to a 1957 paper (38). It was reported (38) that 75 mg of a normal tissue pool contained less HeLa-cytopathogenic inhibiting action than 3.3 mg of the cancer pool used at the same time—a 25-fold difference. Goldstein & Hiramoto (39) reported that in their hands the antiserum supplied to them by Björklund contained no cancer-specific lytic antibody that could not be absorbed by human normal tissues. The discrepancy in results between the two groups appears to this reviewer to rest mainly in the extent of absorption with the normal tissue preparations. Goldstein & Hiramoto, by absorbing as extensively as they did, may have made up for the 25-fold difference in inhibiting action of cancer preparations over normal ones which Björklund's group had obtained.

Other papers in the current literature have also reported the use of absorption techniques in attempts to delineate specificity. Chicken anti-S91 mouse melanoma, after absorption with normal tissue, precipitated an extract of melanoma, and vice versa (40). Antibody against the Yoshida ascites rat sarcoma was completely absorbed by rat and mouse placentas (41). Radioiodinated antibodies against rat lymph nodes and the Murphy-Sturm lymphosarcoma were compared with respect to their ability to be absorbed by insoluble residues of the tissues with the finding that some antigenic components were in common, some not (42). Aimos (43, 44) utilized the absorption technique to study the distribution of H-2 and other antigens in normal tissues and tumors, and uncovered an interesting antigen, alpha, which was apparently shared only by the lymphosarcoma 6C3HED and normal red cells of the strain of origin of the tumor, but not by liver or spleen. One wonders how many other so-called tumor-specific antigens could have been found to have normal tissue counterparts if such care were always taken!

Other cancer antigens.—Some miscellaneous papers pertinent to this discussion have appeared. A so-called specific antigen of human carcinoma was found to be resistant to boiling for 30 min whereas organ-specific antigens were destroyed (45). A qualitative difference between Rous sarcoma and methylcholanthrene-induced sarcoma in chicks was said to exist (46). Human sarcomas and tissues from leukemic patients appeared to have some antigens in common as well as some that were either peculiar to sarcomas or to leukemias (47). Immunological differences were detectable between normal and malignant cells by a fluorescent method (48). Sarcomas induced by benzanthrane or methylcholanthrene contained two distinct types of antigens which reacted with 13 out of 22 rat serums (49). And embryonal muscle and embryonal connective tissue were found to have some antigens in common with human sarcomas which were not found in adult tissues, although there were also antigens common to all tissues and some reportedly specific for tumor (50). The method of rendering animals tolerant to normal antigens while young and of challenging with tumor when adult did not reveal any important antigens specific for tumor (51, 52, 53).

It cannot be too often emphasized that the specificities sought for and

found in this group of investigations are limited to methods used to obtain them, and may be attributable not to chemical differences between normal and malignant tissues but to differences in extractibility or stability or catheptic activity or any one of a large number of other possibilities. It is clear that before an antigen is accepted as being specific for malignancy it must be compared with substances obtained from a battery of normal tissues, of nonmalignant but pathologically involved tissues, and of tissues all the way from embryos to those from aged subjects. There is, of course, no rule that says that a cancer antigen must be completely chemically specific to make it useful; hence, it would not seem necessary for investigators to make extravagant claims at moments of exuberant but uncontrolled discovery in order to justify their findings.

Antigenic loss.—While some groups have been busy looking for specific antigens, others have been seeking a lack of normal antigens. The work of Kay & Wallace (54), for example, demonstrated that in tumors arising from urinary tract epithelium a partial or even complete loss of A and B blood-group antigens occurred. Antigenic loss during carcinogenesis (55, 56) and the poor antigenicity of leukoblasts of lymphocytic leukemia (57, 58) have also been cited as examples of this phenomenon. In an attempt to study antigenic transformations from normal to malignant cells, deSomer & Prinzie (59) have explanted cells from primary growths directly into culture. Since the formation of single-cell clones was not technically feasible in their experiments, selection has been considered by discussers of the paper to be as likely an explanation for the shift in antigenicity as transformation. The subject of antigenic loss due to heteroploidy—loss with respect to the system of substances being measured—has been brought up to date by Hauschka (60).

To study antigenic loss or antigenic gain, a positive knowledge of normal antigenicity is required. The usefulness of isoantigenic systems in such work cannot be overemphasized. Amos (44) has reviewed the work in mice and has tabulated the make-up of 20 phenotypes. Recent investigations by a number of scientists in the areas of "weak" histocompatibility antigens in mice (44, 61, 62, 63), of immunogenetic systems in rats (64, 65), and of new methods of study (44, 66, 67, 68, 69) have certainly been important contributions to the study of cancer antigens as has also the identification of normal constituents such as myosin (70).

Hepatoma antigens.—Investigations of specific antigens in hepatomas have been carried out in a number of ways. The cathepsins of hepatoma 31 and of normal rat liver were immunologically analyzed by Maver & Barrett (71) a number of years ago and found to have distinct antigens as well as some in common. Mouse hepatoma extracts were found by Korosteleva (72) to contain specific antigens not present in soluble liver proteins as shown by sensitization-desensitization-anaphylaxis reactions; in addition, antisera against the hepatoma gave stronger precipitin reactions with water extracts of the hepatoma proteins than with those of normal liver.

In an outstanding series of experiments by Zilber (73, 74), Abelev & Avenirova (75, 76), and Abelev & Tsvetkov (77), two separate antigens were identified and isolated, one specific for mouse hepatoma and the other for mouse liver. The organ-specific antigen of mouse liver, a lipoprotein called AO, was found in the fairly insoluble fraction of liver-cell granules and could not be found in hepatoma, kidneys, lungs, or spleen. The hepatoma antigen was salted out by ammonium sulfate, purified further by zone electrophoresis on agar, and identified by a specific antihepatoma-antibody reagent which had been eluted from an antibody-hepatoma-protein complex. The extract, still contaminated with normal tissue antigens, was separated from the agar and subjected once again to electrophoresis, but this time against a counter-osmotic stream of globulin which contained antinormal-liver and antinormal-serum antibodies. The normal tissue antigens were thus precipitated or their migration reduced as they met the antisera, while the specific hepatoma antigens which continued to migrate became available in a highly pure form.

Yet another method for observing specificity in rat hepatomas was carried out in the author's laboratory (78, 79). Radiolabeled antihepatoma antibodies were assayed *in vivo* in autochthonous hepatomas induced by N-2-fluorenylacetamide. By the method of absorption *in vivo* in normal rats and specific purification from hepatomas, preparations of radiolabeled antibody were obtained of which 40 per cent localized in hepatomas. Only 4 per cent localized in normal liver of normal rats, and none localized in other tissues. The observed localization specificity was not necessarily indicative of chemical specificity since this type of antibody assay depended upon accessibility of antigens to the circulation.

Isograft antigens.—The isograft method of detecting specific cancer substances does not require isolation of the substance or preparation of an antiserum reagent. Part of the isologous cancer to be tested is heavily irradiated and used to "sensitize" the host, and part is used as the isograft to "challenge" the host. Isograft rejection is the index of specificity. Révész (80) discovered antigenic differences between methylcholanthrene-induced mouse sarcomas of recent origin and their isologous hosts, but found complete compatibility between spontaneous carcinomas or lymphomas of recent origin and their hosts. Prehn (81) discovered a similar specificity, not only in methylcholanthrene-induced fibrosarcomas but also in dibenzanthracene-induced tumors. Normal spleen from the same mice failed to induce immunity when tumors were used as challenge, and normal skin failed to be rejected as a challenge isograft when tumors were used to sensitize. Klein *et al.* (82, 83) were concerned about the control for heterozygosity and therefore used the same animal for host in which a leg-tumor had been induced by methylcholanthrene. After tumor induction and leg amputation, part of the tumor was irradiated and used to "sensitize" the host; part was carried isologously until needed for a challenge inoculum. Out of 16 mice so challenged with their own tumor, 12 were resistant. Resistance was relative since, with very high

cell doses for the challenge, the resistance barrier could be broken. Isologous mice treated in the same way as the autologous mice displayed greater resistance more frequently. Even when solidly resistant to high cell doses of one sarcoma, however, such isologous mice could be challenged with low cell doses of another sarcoma and promptly succumb. Klein *et al.* (82) state the problem by asking a question: "What is the explanation for the paradoxical finding that antigenically foreign-cell clones can develop into a tumor in an animal and are not automatically eliminated by the immune response?"

Viruses as antigens.—Although viruses which grow specifically in cancer may be thought of, in a sense, as cancer-specific antigens, the usefulness to which antibodies against cancer-specific viruses can be put in the immunological control of cancer may be limited by the paradox of Sjögren, Hellström, & Klein (84):

Whereas mice inoculated with polyoma-virus as newborns develop high titers of antiviral antibodies, they cannot prevent the development of their own polyoma-induced primary tumors, although animals immunized as adults prove to be resistant against the transplantation of established polyoma-tumor cells.

The resistance of virus-immune mice to polyoma-derived transplanted tumors was not only observed by Sjögren *et al.* (84, 85), but also by Habel (86) and by Sachs (87). The development of high antiviral titers in a variety of animal species after virus-tumor induction has been noted by many investigators, some of the more recent reports being those of Fogel & Sachs (88), Roizman & Roane (89), and Bergs & Groupé (90).

Immunological techniques have been very successfully used in a number of ways for detecting viruses. Fluorescent antibodies have been used to measure polyoma-virus synthesis in tumor cells (91) [although Dulbecco (92) is unwilling to accept the thesis of synthesis in that case] and for detecting the mammary-tumor agent and the Shope, Rous, and polyoma viruses in tissues and cells (93, 94, 95, 96). The antibody inhibition of the virus-hemagglutination reaction has been used to measure the distribution of virus in mice (97); tolerance, to render ducks susceptible to the Rous sarcoma virus (98); the sensitization-desensitization-anaphylaxis method, to show differences between Shope papilloma virus and extracts obtained from virus-induced tumors (99); the gel-diffusion technique, to demonstrate virus-induced changes in lactating mammary glands of high and low cancer strains of mice (100); and Ovary's passive cutaneous anaphylaxis technique, to demonstrate the similarity between viruses found in mouse and human leukemias (101).

The X antigens.—Amos, Gorer, & Mikulska (102), while analyzing the antigenic structure and genetic behavior of the transplanted leukemia, E.L.4, found an unusually complex situation in the case of mice protected against the tumor with isoantisera. Having observed that protection by passive immunity could be accomplished even in the home strain of mice, C57BL, Gorer & Amos (103) felt they had found a specific tumor antigen which they called "X." "Was this a special case or a common feature of leukemias?"

Gorer asked (104). The answer was found in three other leukemias in which passively administered isoantibody was found to protect susceptible hosts [Amos & Day (105)]. Before his untimely death Gorer (106) extended the study to four more leukemias of the E.L. series, 5, 6, 7, and 8, and concluded:

Both spontaneous and induced leukemias have X antigens. Even within a closely inbred sub-strain of C57BL each leukemia had a different X component although some cross reactions were found. It is not known if the X antigens are qualitatively different from any normal component or if the difference is merely quantitative.

He concluded, however, that even a quantitative difference of sufficient magnitude might form a useful target for chemotherapy and that, therefore, it would seem worthwhile to study the chemical nature of the X antigens. He might have added that the same would appear to be worthwhile for cancer antigens in general.

THE TUMOR-HOMOGRFT REACTION

Although few now regard the tumor-homograft reaction as one that is directly parallel to such autoimmune processes as spontaneous regression of autochthonous cancer, many do turn and return to the study of cancer-graft phenomena as a method of observing the interplay between humoral and cellular immune forces, and discovering their modes of action. Gorer lists three histologically distinct types of primary response of a host to tumor homografts in mice (107, 108): (a) fibrovascular stromatization of a graft by the host and ultimate invasion by cells of the lymphocyte series; (b) growth without the host's stroma such as in ascitic fluid, in a subcutaneous space, or in the gastrocnemius muscle, and destruction intimately associated with histiocytic activity; and (c) extensive exudation of plasma in which histiocytic invasion is a late phenomenon occurring only after graft destruction is advanced. The secondary responses which follow the three types of primary response, after the hosts have rejected their first grafts and are rechallenged, are likewise distinctive.

Cellular antibody.—The importance of cellular antibody to the tumor-homograft reaction was made clear after Mitchison's experiments (109, 110), which showed that passive transfer of transplantation immunity could be accomplished with lymph nodes regional to a former incompatible graft in another host even when passive transfer could not be demonstrated with humoral factors. This technique of adoptive immunity has since been utilized in a number of situations to demonstrate that a host had immunologically reacted to a graft even if it had not produced detectable humoral antibodies as proof. A recent example is found in the work of Winn (111, 112) who has been developing methods for the quantitative assay of immunologic activity of lymphoid cells stimulated by tumor homografts in mice. Evidence was obtained that tumor and lymphoid cells do not interact *in vitro*, that lymph-node cells are immunologically immature at the time of excision but do mature in new hosts, and that lymph-node cells, when mature, do indeed

attack target-tumor cells directly and not by elaboration of serum-borne antibody. Rosenau & Moon (113) reported, furthermore, that lymphocytes of BALB/c mice, sensitized to L cells (C3H origin), were seen to cluster around L cells in synthetic tissue-culture media and cause cytolytic effects in the absence of complement or demonstrable antibody.

The reports of Wiggzell's adoptive immunity experiments (114) tend to modify the concepts of directly acting lymphoid cells. F1 hybrid mice, when injected intraperitoneally with lymphoid cells of one parental strain, may be shown to be resistant to subcutaneous growths of lymphomas from the other (but not the same) parental strain. The reaction suggests that humoral antibodies may be formed *in vivo* by lymphoid cells of the one parent and protect against tumor cells from the other. There is little if any effect upon carcinomas, sarcomas, or skin grafts, but the absence of a reaction does not prove the absence of antibody in such cases, only that antibody, if present, is ineffective.

In an excellent critical review of the immune response and the homograft reaction, Winn (115) points out what many of us seem to have forgotten—that an animal may display a high degree of specific resistance in the absence of an appreciable level of serum antibody simply because it is in a position to make a good secondary response. Cellular transfer of immunity, although usually accomplished with lymph-node cells, can also be brought about by spleen cells, but not necessarily by all lymphoid cells of the host. As Mazarek & Duplan (116) have shown, bone-marrow cells are poor vehicles for the transfer. Snell, Winn, & Kandutsch (117) also noticed variations in active cellular immunity among the various lymphoid organs. These studies and many more will be needed before we can approach future immunotherapy experiments with understanding. As Albert & Podolak (118) showed, the lymph nodes, regional (and otherwise) to spontaneous tumors in mice, appear to contain no clues to the rôle they might be playing in autochthonous neoplasia.

Humoral antibody.—Humoral antibody has not been discarded as an unimportant factor in tumor-immunity concepts. For one thing, humoral antibody is not a species but a state of being, and many immune reactions ascribed to cells may, in fact, be due to "humoral" antibody carried or elicited by cells. The fact that antibodies are so much less cytotoxic *in vivo* than *in vitro* is a stumbling block, however, to our understanding of their action. Amos & Wakefield (119) found, for example, that the recovery of a population of ascites tumor cells, located intraperitoneally in diffusion chambers in mice, could be delayed indefinitely by repeated injections (outside the chamber) of isoantisera, but, unlike their behavior *in vitro*, could not be completely lysed; availability of complement seemed to be a limiting factor. In later experiments the same team (120) found that, before ascites tumor cells are rejected by an incompatible host, antibodies have already appeared in the serum. In addition, even while the tumor is growing, an increasing number of tumor cells become sensitized, and these sensitized

cells are killed *in vitro* by addition of complement without added antibodies.

It is still rather difficult to fit many observations into a pattern in view of phospholipids that inhibit complement (121), antibodies that do not need complement (122), natural cytotoxicins that require complement (123), and antigens that do not elicit complement-fixing antibody (68). Unabashed by such confusing reports, Amos (124) has gathered together a most admirable series of hypotheses to describe the relationships between the cytotoxic effects of iso-antibody and host-cell functions. Four mechanisms are offered as possibly operative in the homograft reaction: (a) direct lysis, (b) phagocytosis, (c) ischemia and fibrosis, and (d) massive enzymatic destruction. The enzyme attack would be triggered by antigen from the graft which coats host cells and permits antibody plus complement to lyse them. Proteolytic and oxidative enzymes, thus released, would kill and lyse other cells including those in the graft; fresh antigen from the graft would perpetuate the reaction.

Immunological enhancement.—"The outcome of inoculations is determined by an interplay between the hostility of the host and the proliferative vigor of the implant, hence an absolute immunity does not exist." These words of Woglom (125) are best illustrated by a well-known but little-understood homograft phenomenon which involves humoral antibody—that of immunological enhancement. As Gorer (126) has stated,

Many of us have unburdened ourselves of theories of enhancement. I plead guilty; Medawar has done it; Snell has done it; and Kaliss has done it . . . , but all of us, I think, would agree on one thing: that somehow humoral antibody can interfere with the proper function of the host cells.

The rôle of classical immune forces was not appreciated until Kaliss, Molomut, Harriss, & Gault (127) used hetero- and iso-immune serums to produce the effect by passive immunization and until, subsequently, Kaliss & Day (128) showed that active immunization of the host would persist ten months or longer. Interest then mounted in the enhancing substance which was eventually shown to be extractable in active soluble form by a butanol-water system (129) and by Triton (130). Since normal tissues were found to share these substances with tumors (131), interest has since shifted to the enhancing antibody and to the tumors that are enhanced (132). Gorer & Kaliss (133) found that the dose of antiserum was important when a tumor such as the B.P.8 sarcoma, crossed strain barriers: high serum doses resulted in inhibition; lower doses, in enhancement. With sarcoma 1 only enhancement was observed; with E.L.4, only inhibition. It is interesting to recall in this connection an early experiment in which Kaliss (134) had shown in still another tumor-host system that a high dose of antigen led to enhancement; a lower dose, to inhibition. Winn (115) does not regard enhancement as a stimulation of tumor to grow beyond its normal rate (as defined by its growth in compatible hosts where it grows even faster) but as a depression of immune responses of an incompatible host. One way of depressing an active immune

response according to Uhr & Baumann (135) is to administer antibody by passive transfer, and they feel immunological enhancement should be viewed from this aspect. It is difficult for this reviewer to conceive of such a mechanism for enhancement in view of the dosage effects shown by Gorer & Kaliss and by Feldman & Globerson (136). The latter group has actually demonstrated enhancement of tumors with heteroimmune antibody while isoantibody was being produced by the host. Winn, however, may still be right since there is one other way of possibly depressing an immune response—that of lessening the antigenicity of graft cells; e.g., by coating them with antibody. Since by decreasing antigenicity one must also be lessening the ability of the graft to induce a foreign-body response, one may in immunological enhancement be no more than decreasing the irritability of a graft to a low level. A certain amount of irritation is probably necessary to evoke a fibrovascular stroma reaction or to initiate angiogenesis. Decrease of the irritability below this level with large amounts of antibody, as in the case of B.P.8, would prevent the tumor from gaining a foothold in the first place. It would appear, therefore, that immunological enhancement could be thought of as being sandwiched between two types of immunological inhibition, type one occurring after and type two before a vascular stroma had developed.

The phenomenon of drug-induced tumor-homograft acceptance by an otherwise resistant strain, which has been briefly reviewed by Uphoff (137), has been commonly termed immunological drug-induced tolerance (137, 138, 139). The drugs are those which in compatible host-tumor combinations often result in graft rejections—e.g., by 6-mercaptopurine and amethopterin and some of their derivatives. The action of the drug in the incompatible host has been thought to be a suppression of antibody response, hence a type of tolerance, but evidence is accumulating to indicate that the hosts have actually undergone an immune response (140, 141); moreover, the drugs apparently do not interfere with the ability of animals to make antibodies against "pure" antigens (139). Perhaps it may turn out that the pertinent action of the drug is actually to lessen the local foreign-body response of the host to the graft. In an otherwise incompatible host this would result in bringing the high irritation level of the graft down to a level necessary for growth, hence, from type-one inhibition to acceptance; in an otherwise compatible host this would result in bringing the irritation level below the threshold necessary for growth, hence, from acceptance to type-two inhibition.

Breyere & Barrett (142, 143) and Prehn (144) have described another technique for bringing about compatibility in a host to skin and tumor homografts—that of parity in which females, after repeated matings with males of another strain, become conditioned to accept otherwise incompatible grafts from mice of the same strain as the male. The authors, apparently on the basis of the skin-graft work, refer to parity as an example of immunological tolerance. Tests for the absence of antibody production against the grafts have not been carried out, to this reviewer's knowledge. It does seem pos-

sible, therefore, that a state of immunological enhancement may have been evoked instead of tolerance. Breyere & Barrett (143) point to the strange behavior in the growth of the plasma-cell tumor grafts in the "tolerant" hosts as attributable perhaps to the development of local immunity. If this explanation be true, then tolerance would be even less commensurate with the facts, whereas enhancement could still form the basis of a satisfactory explanation.

IMMUNOTHERAPY OF AUTOCHTHONOUS CANCER

Preventive immunity.—Attempts to ward off the onset of autochthonous cancer by preventive immunotherapy, active or passive, of animals or humans have not been very extensive, and those few attempts have always resulted in failure. It has been known since the days of Clowes and of Ehrlich that animals can be made immune to transplanted cancers by either active or passive immunotherapy. It was because of such heartening findings early in the annals of cancer immunity that Gaylord (1) declared that cancer is a curable malady. Gross (145), in a simple experiment described in an infrequently cited paper, revealed the contrast between preventive autoimmunity and preventive transplantation immunity; inbred C3H mice, which had been injected with methylcholanthrene-induced transplantable sarcomas and which had subsequently been found to be resistant to such transplants, nevertheless developed spontaneous mammary tumors. In a similar experiment, Mol'kov (146) immunized isologous rats against transplanted tumor material and showed them to be continuously nonsusceptible to the same transplanted tumor for six months as well as immune to another transplanted tumor. Yet the same rats failed to show immunity to tumors induced by methylcholanthrene which appeared at the same time as in the controls with the same incidence, morphology, and histology. In a second series, the same experimenter (147) failed to immunize rabbits against carcinogenesis by prior injections of transplanted tumor material. In the hands of one group of investigators (148), some survivors of an inoculation of sarcoma 180 actually developed a lymphocytic leukemia.

In a previous section it was reported that Prehn (81), Klein (82), and Day (78) had all demonstrated the presence of possibly cancer-specific antigens in tumors induced in animals in their respective laboratories. Yet Prehn (149) failed to immunize mice against dibenzanthracene carcinogenesis; Klein *et al.* (82) failed to immunize mice against methylcholanthrene carcinogenesis or alter the course of appearance or incidence of tumors; and Day & Planinsek (150) failed to immunize rats against N-2-fluorenylacetamide carcinogenesis or alter time of appearance, incidence, or site of involvement. Hirsch and Iversen (151) did not merely fail to obtain active preventive immunity in C3H and C3H-backcross mice to spontaneous mammary tumorigenesis, but, in fact, obtained evidence of accelerated development of the tumors. Graham *et al.* (152) summarized the apparent view of cancer clinicians and surgeons

to preventive immunotherapy in humans. When asked if he had plans to use his cancer vaccine on well persons to see if it prevented cancer, as opposed to its use to cure cancer that has already occurred, Graham's answer was simply, "No."

Curative immunotherapy.—Immunotherapy, active or passive, of established autochthonous tumors in animals apparently has not been a subject of interest to experimentalists, and, to this reviewer's knowledge, there has been only one paper on the subject (to be discussed at the end of this review). Perhaps the reason is again contained in the views of Graham *et al.* (152) who declared: "Immune therapy of human cancer must be studied and developed in patients. Transplantable tumors are valueless and even spontaneous tumors in animals are dubious counterparts of the disease in people." The charge that transplanted tumors are valueless for immunotherapy studies should be well taken by all of us; on the other hand, the second charge, that even spontaneous tumors in animals are dubious counterparts of the human disease, should be challenged. There has not been enough work with such tumors to prove the matter one way or the other (153). Hirsch (154) thinks the use of spontaneous tumors in animals for immunity experiments should be advocated, but not the use of chemically-induced tumors. Mol'kov (155) takes a middle-of-the-road view of the subject in his review on the problem of specific autotumor vaccination. He concludes that although there is still no serious use of preventive vaccination against cancer, investigations on active immunotherapy of established cancers should be carried out "with induced and spontaneous tumors of animals of inbred lines and with human tumors."

Curative therapy by passive immunity.—Passive immunotherapy, proposed by Domagk (156), has been taken seriously by only a few investigators (157, 158). Most of them believe, with Milgrom (5), that serotherapy of solid tumors is not promising enough at present to warrant investigations in humans. Pressman (159) felt that an anticancer antibody could be made cytotoxic by carrying with it enough radioactivity to irradiate the tumor. It was with this view in mind in 1952 that he began, with Korngold (160), the work on radiolabeled antitumor antibodies that has since been carried out in a number of laboratories; however, as has now been shown (78), all work on the localization of radiolabeled antibodies in solid transplanted tumors must be held suspect as merely due to the cross-reaction of antibodies with fibrinogen in the circulation and subsequent localization in tumors as passive partners of rapid fibrin deposition. [Reports of localization of labeled antibody directly on ascites cells (161, 162) do not need to be held with the same suspicion.] It has recently been shown that cross-reacting labeled heteroantibody, other than antifibrin antibody, will localize *in vivo* in autochthonous tumors, induced hepatomas in rats being the target (163, 164). Specific localization of labeled heteroantibody in such tumors (78, 79) has also been obtained, but only time will tell whether such an antibody can be used immunotherapeutically.

Localization of antifibrin antibodies has not been seen to occur except on rare occasions, and then perhaps nonspecifically, in autochthonous tumors of rats or dogs (163, 164, 165); however, to illustrate the use to which radio-active specific-localizing antibody might be put in immunotherapy, Bale, Spar & Goodland (166) localized highly radioactive antifibrin antibodies in the Murphy-Sturm lymphosarcoma and caused rapid and permanent regressions of the transplanted tumors in the rats with a minimum of radiation damage to the subjects.

Curative therapy by active immunity.—Many investigators believe that immunotherapy, if it is realized, will come from the realm of autoimmunity. The rationale of this approach was stated clearly and simply by Milgrom (5): "If proper methods of immunization may result in the destruction of normal organs, why would it not be possible to achieve the destruction of a tumor in a similar manner?" The proper methods of immunization obviously have not yet been found. Some systematic work on methods of immunization is being carried out (167, 168, 169). More work is needed. It is interesting to note that although interest in the relationship between the nervous system and immunogenesis is high in Russia (27, 170), a recent collection of papers on the neoplastic process and the nervous system (171) did not include any discussions of tumor immunity as influenced by the nervous system.

The vaccinations of cancer patients by Witebsky and his colleagues (172), Fediushkin (173), Trapeznikov (174), and Graham and his associates (152), have all been ineffective. But, in the words of Mol'kov (155): "Trials of specific vaccination of human patients after surgical ablation of primary growths must be continued." Because of the high degree of anergy in many cancer patients such as in those with Hodgkin's disease (175), the problem may be, as Graham *et al.* (152) have outlined, to make cancer patients less tolerant and more reactive. On the other hand, it may not be even that simple, for, as Murphy & Syverton (176) have shown for mice, susceptibility to leukemia is not related to immunological capacity. Interest in spontaneous regressions is high (177, 178) because of the clues that such regressions may offer to bring about successful immunotherapy of other cancers.

Other clues are found in accounts of circulating autoantibody or autoantigen, or both, in cancer patients (179, 180, 181, 182), the presence of delayed hypersensitivity in others (183), the occurrence of auto-hypersensitivity (184, 185, 186), an increase in cancer patients of a plasma factor which reacts with antigens from their tumors (187), and the presence of anomalous plasma proteins in cases of autochthonous cancer (188, 189, 190). Speculative (191, 192), editorial (193), and critical (5, 181, 194, 195) reviews of the subject notwithstanding, only vague meaning has so far been obtained from such clues relevant to immunotherapy.

Lumsden's forgotten experiment.—There is one paper which pertains to the subject of sero- and immunotherapy of established autochthonous cancer in animals which has never been included in any of the popular reviews of tumor immunity since the day of its publication in 1932. In this forgotten

paper, Lumsden (196) describes how he injected euglobulins (from sheep antihuman- or sheep antirat-cancer serums and from normal sheep serum) directly into spontaneous mammary tumors arising in Loeb-Lathrop-Simpson strain (Marsh strain) mice. Several days later, he relates, the tumors were partially excised and autografts made. (The normal euglobulin had had no effect on the tumors but the immune globulins had caused partial destruction in 40 mice, and complete and rapid regression in seven.) Two measures of immunity were then employed: whether the autografts took and whether tumor recurred at the primary site. Because of the euglobulin treatment the tumors were also grafted in normal isologous mice to check for viability. Table I reveals the results.

TABLE I
IMMUNOTHERAPY OF ESTABLISHED AUTOCHTHONOUS CANCER IN ANIMALS

Measure of Immunity		Number of Mice	
Autograft growth	Local recurrence	Experimental (anticancer serum)	Control (normal serum)
—	—	27	0
—	+	4	1
+	—	2	16
+	+	7	23

On the basis of Lumsden's results, immunotherapy of established autochthonous cancer in animals, a most neglected field, would appear to contain more promise for the future than any other single approach.

Additional background material on various aspects of tumor immunity.—Cloudman (197); Ebert & DeLanney (198); Gorer (199, 200); Hauschka (201); Klein (202); Klein & Klein (203); Lumsden (204, 205); Radzikovskaia (206); Snell (207, 208); Spencer (209); Woglom (210); and Symposium sponsored by the American Cancer Society: *The Possible Role of Immunology in Cancer* (211).

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INBORN ERRORS OF METABOLISM^{1,2,3}

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The rapid growth and progress of this subject are evident from the number of articles which have appeared (1, 2, 3) and which have been discussed (4), reviewed (5, 6, 7, 8), and codified (9, 10). In the present review, recent developments within the general framework of metabolic defects in man at or near the enzyme level are considered. A certain degree of interest and prejudice on the part of the reviewer will be apparent in the selection of material.

Inborn metabolic errors have served to highlight knowledge of metabolic pathways and focus attention on transport, as well as on control mechanisms. In the future, it is anticipated that genetic diseases will continue to influence even more profoundly the thinking in control mechanisms, including hormonal control where several sites or expressions of control appear to be emerging. To treat, in this review, the metabolic as well as the functional aspects of the subject, diseases are grouped biochemically, and then functionally.

GLYCOGEN STORAGE DISEASES

Phosphorylase defects.—The multiple nature of the enzymic defects of the glycogenoses was established by the late G. T. Cori and co-workers (11). Recent work enlarged the original four groups to six (12). McArdle's disease (13), a myopathy associated with glycogen storage, is now classed with the glycogenoses (Type V) or with the diseases of muscle (14). Here the enzyme defect was established as an absence of phosphorylase (15, 16, 17). The presence of Urd-5'-P-P-Glc² pyrophosphorylase (17) and Urd-5'-P-P-Glc glycogen transglucosylase (15, 16) provided strong evidence for the anabolic role of the irreversible uridine-linked sequence of enzymes (18). From immunologic studies, Robbins (19) concluded that the phosphorylase protein in this disease is either completely lacking or is so modified that it does not cross-react with antibodies. The fact that muscle glycogen content is only minimally increased with so severe an enzyme defect indicates that another

¹ Most of the literature cited in this review appeared before January 1961.

² The following abbreviations are used: Urd-5'-P-P-Glc (uridine diphosphate glucose); Pi (inorganic phosphate); NADP (nicotinamide adenine dinucleotide phosphate); NAD (nicotinamide adenine dinucleotide).

³ I wish to express appreciation to many investigators who supplied experimental information before publication, or in press; and to colleagues at Western Reserve University for helpful discussion, suggestions, and criticisms. I wish to thank Miss H. Sasko for aid in the literature search, and for the help in the final stages of completing the manuscript.

facet of the disease is one which limits the overall accumulation of glycogen (15). Possibilities to be considered include breakdown by hydrolytic enzymes such as amylase (20, 21) and decreased extraction of blood glucose by muscle (22). Schmid & Hammaker (23) provided a family study and used the failure of blood lactate to rise after ischemic exercise to demonstrate a recessive mode of inheritance.

Hers (24) first described glycogen storage in liver associated with diminished phosphorylase activity (Type VI). The complex nature of this disease (or these diseases) was recently pointed out (25). In one family, three children were affected: one had decreased phosphorylase activity with low glycogen content, the other two had phosphorylase activities which were not decreased but had increased stores of liver glycogen. One might again speculate as to whether glycogen breakdown via a nonphosphorolytic pathway (26, 27) might become of sufficient quantitative importance over a period of time to limit the overall accumulation of glycogen, and thus explain the normal glycogen content of the first case. The other two cases point up the question of a defect in the phosphorylase protein (or activating system). The protein formed might appear normal when assayed *in vitro* under nonlimiting substrate conditions but yet be a less-effective catalyst *in vivo*. Enzyme purification and characterization studies have proved difficult because of the small amount of biopsy material available (25). These questions may be suitable ones for approach through tissue culture techniques (28). An increased content of erythrocyte glycogen was noted in this form of the disease [Sidbury, Gitzelmann & Fisher (29)].

Branching and debranching defects.—A second case of an abnormal glycogen structure resembling amylopectin was described (29). Liver, kidney, and muscle as well as blood contained an unusual polysaccharide with an increased average outer chain length (Type IV). A branching-enzyme defect is presumed in this type.

Hers (25) devised new techniques for measuring amylo-1,6-glucosidase. One was based on an observation of Larner & Schliselfeld (30) who had noted that the enzyme catalyzed a limited reincorporation of ^{14}C -labelled glucose into the phosphorylase limit-dextrin. As a result, it was shown that the disease was present with enzyme defects in liver and muscle in one variant or with a defect in liver but not in muscle (25) in another variant. In both subtypes, erythrocyte glycogen was increased in amount, and decreased in average outer chain size. The explanation of the two variants offers a challenge. The reviewer, in collaboration with Oliner & Schulman (31), recently identified this enzyme defect in liver and muscle biopsy material from a 50-year-old male in whom the disease was associated with myopathy.

Glucosidase defect.—No enzymic basis has heretofore been advanced for Type II (cardiac type or generalized glycogenosis). Hers (25) recently described a defect in the hydrolysis of maltose to glucose (α -glucosidase) in liver and heart. Although the function of this enzyme is not clear at present,

this finding would suggest a role for the hydrolytic pathway of glycogen breakdown through the oligosaccharides (26, 27). An alternative explanation involving a transglucosylation reaction immediately prior to the debranching reaction based on the work of Walker & Whelan (32) was suggested. After structure analysis, Walker & Whelan demonstrated a symmetric rather than an asymmetric deposition of glucose residues about the α ,1-6 link of a phosphorylase limit-dextrin. To explain the release of free glucose from the dextrin by amylo-1,6-glucosidase they postulated an additional step, in which a transglucosylase converted the symmetric to the asymmetric structure. Since these structure studies were done with a phosphorylase limit-dextrin which had been degraded to a somewhat smaller extent than that previously prepared and studied by Cori & Larner (33) (28 as opposed to 36 per cent), they are therefore subject to scrutiny in terms of incomplete phosphorylase degradation. Larner (34), in his studies on glycogen, and recently Illingworth, Brown & Cori (35), with amyloheptaose, demonstrated that crystalline muscle phosphorylase catalyzed a transglucosylation of a randomizing type in the presence of catalytic amounts of Pi .² Illingworth, Brown & Cori (35) showed that amylo-1,6-glucosidase free of detectable transglucosylase activity still released glucose from the phosphorylase limit-dextrin. From these and other studies with α -amylase (36) it appears that differences in dextrin structure may depend on extent of enzymic degradation. The role of the transglucosylase in glycogen metabolism is apparently not clarified at the present time.

Glucose-6-phosphatase defect.—The important direct and indirect roles of glucose-6-P in metabolism are indicated by the variety of metabolic effects associated with an absence of glucose-6-phosphatase (Type I). In 1943, Mason & Sly (37) first noted that blood lactate was elevated, and they suggested that the lactate was formed as a consequence of glycogen breakdown. This would result from a deficiency in the dephosphorylation of hexose monophosphate to blood glucose and its conversion to lactate instead. Since lactate was shown to decrease renal urate clearance in normals (38) Howell, Ashton & Wyngaarden (39) suggested that this decreased clearance may explain the marked increase in blood urate observed in three siblings with the disease. Glucagon raises blood lactate according to Sokal, Lowe, Sarcione, Mosovich, & Doray (40). Further, hepatic glycogenolysis was increased during fasting, but with glucose administration glycogen was deposited, with decreased blood lactate. From these studies, they concluded that liver was the chief organ source of blood lactate. These studies must be interpreted cautiously in view of the recently demonstrated action of glucagon on the isolated heart [Farah & Tuttle (41) and Cornblath, Morgan & Randle (42)].

Since no alteration in erythrocyte glycogen content has been noted (29), it is surprising that increased levels of erythrocyte glucose-6-P have been reported (43). With the demonstrated stimulation of liver Urd-5'-P-P-Glc glycogen transglucosylase by glucose-6-P (44), increased hepatic levels of

this sugar phosphate in contact with the enzyme would provide an attractive explanation for the high liver glycogen levels (45). The remarkable apparent stability of the central nervous system in the absence of demonstrable blood glucose is as yet unexplained (46).

Multiple defects.—Sokal, Lowe, Sarcione, Mosovich & Doray (40) noted a deficiency of both phosphorylase and glucose-6-phosphatase in one patient. Cases of combined deficiencies in amylo-1,6-glucosidase and glucose-6-phosphatase have also been described [see (29, 47)]. The sibling of a patient previously shown to have an amylo-1,6-glucosidase defect was shown to have glucose-6-phosphatase defect (48). Since it is difficult to separate genetic from post-genetic events, it is clear that generalizations regarding genetic mechanisms would appear to be premature at the present time (29). Of interest in this connection are the findings of Kalckar, Kurahashi & Jordon (49) who identified several *Escherichia coli* K-12 mutants with multiple defects in the Leloir galactose pathway.

Diagnosis.—The enzymic analysis of biopsy material appears to remain the single technique of greatest significance. Considerable information, however, has been gained from blood enzyme and glycogen studies (29). Of the indirect methods, fructose- and especially galactose-tolerance tests proved of value (46). Similarly, lack of rise of blood lactate after ischemic exercise proved useful (23). Increased serum aldolase and transaminase activities were noted with liver glycogenoses (50). They appear related to liver diseases in general (see below).

GALACTOSEMIA

Elucidation of the galactose-1-P uridyl transferase defect was reviewed by Kalckar (28, 51). The importance of the discovery of the accumulation of galactose-1-P in erythrocytes, as well as the apparently normal development of affected infants on galactose-free diets, were key observations which suggested the specific enzymic site. Kirkman & Bynum (52) used an enzyme assay coupled to methylene-blue oxidation and showed that parents of affected children had lower than normal enzyme activity in erythrocytes. They were able to relate enzyme activity to three genetic states, nongalactosemic homozygous, heterozygous, and affected homozygous. Huang, Hugh-Jones & Hsia (53) and Bretthauer, Hansen, Donnell & Bergen (54, 55) made similar observations. Schwarz, Holzel & Komrower (56) incubated cord erythrocytes in galactose and measured the increased accumulation of galactose-1-P as a means of early detection.

Chronic organ toxicity was discussed in terms of accumulated galactose-1-P by Holzel, Komrower & Schwarz (57) and by Sidbury (58). The latter worker (59) and Ginsburg & Neufeld (60) reported that phosphoglucomutase was inhibited by galactose-1-P. Cusworth & Dent (61) discussed the reversible aminoaciduria, which was originally noted in this disease by Holzel, Komrower & Wilson (62). It was pointed out that the renal tubular disorder is a general one, which may include glucosuria as well as proteinuria, and

which does not appear to be related directly to galactose, in keeping with the galactose-1-P inhibition hypothesis.

Lerman (63), who had demonstrated the enzyme defect in cataractous human lens, measured a decreased glucose-6-P dehydrogenase activity in homogenates of rat lenses prepared from animals which had been placed on a high galactose diet (64). Since the enzyme was inhibited by added galactose-1-P *in vitro*, it was suggested that galactose-1-P might inhibit the enzyme *in vivo* and thus limit the energy supply through the pentose phosphate pathway. Weinberg & Segal (65), on the other hand, found that they were unable to demonstrate an inhibition of the pentose phosphate pathway, studied by oxidation of ^{14}C -1 and ^{14}C -6 labelled glucose to $^{14}\text{CO}_2$, although galactosemic leucocytes contained increased levels of galactose-1-P when incubated in galactose. Of considerable interest is the recent finding of Elder, Segal, Maxwell & Topper (66), who showed that the capacity to oxidize a tracer dose of ^{14}C -galactose to $^{14}\text{CO}_2$ was greatly increased when progesterone or menthol was administered to galactosemics; this suggests a bypass of the block. These findings follow up, and may be related to, the observation of Topper [see (67)] that steroids exert a marked stimulating effect on galactose oxidation in liver slices.

FRUCTOSE INTOLERANCE

A congenital disease with severe clinical manifestations, including hypoglycemia on ingestion of fructose and differing from benign fructosuria, was described by Chambers & Pratt (68). Froesch, Prader, Wolf & Labhart (69) noted a rapid and marked decrease in serum inorganic phosphate accompanying the decrease in blood glucose, after intravenous fructose administration. The drop in inorganic phosphate was attributed to its binding by fructose during formation of fructose-1-P. A decreased utilization of fructose-1-P would account for the slow release of inorganic phosphate. A block at the level of fructose-1-P aldolase was therefore postulated. Hers & Joassin (70) recently demonstrated a decreased and modified liver aldolase activity in two patients with the disease. No decrease of liver fructokinase was noted. With fructose diphosphate and fructose-1-P as substrates, activity was reduced to about 25 per cent and 4 per cent, respectively, of control values. The fructose diphosphate to fructose-1-P activity ratio was about six times higher than the ratio shown by controls. Hers discussed the possibility of a single mutation modifying two enzymes or affecting one enzyme that is active with both substrates. The finding that liver aldolase of premature infants had a fructose diphosphate to fructose-1-P activity ratio 2 to 5 times that of normals after birth suggests that the fetal enzyme may differ from the adult, and that the disease may be related to the presence of a fetal-type enzyme. Since liver fructokinase was inhibited by fructose-1-P *in vitro*, the hyperfructosemia and fructosuria may be explained on this basis. Hypoglycemia seems not to be attributable to pancreatic insulin release and may be related to inhibition of phosphoglucomutase by fructose-1-P (69).

PENTOSURIA

Touster (71) and Knox (72) reviewed this disease. Despite the lack of direct enzyme studies, excretion of L-xylulose and of the recently discovered L-arabitol (73) provide strong presumptive evidence for localizing the block at the level of the NADP-linked L-xylitol dehydrogenase (71). The *in vivo* work of Hiatt (74), demonstrating the conversion of labelled glucuronolactone to L-xylulose but not to ribose in the pentosuric, provides strong substantiation. The question, raised by Knox (72) of a possible renal mechanism appears to have been answered by the recent experiment of Bozian & Touster (75). They showed that administration of glucuronolactone to pentosurics gave rise to marked increases in plasma xylulose levels, in contrast to the levels attained by nonpentosurics. Detection of carriers by a glucuronolactone load test was described by Freedberg, Feingold & Hiatt (76).

Margolis' (77) original curious observation that aminopyrine increased pentose excretion in pentosurics, but not in normals, provided a springboard for studies on the mode of action of drugs which increase the excretion of various members of the glucuronate-xylulose cycle. Touster (71) summarized these observations and suggested different sites of action for borneol (and probably menthol) from chloretone, aminopyrine, barbital, and carcinogenic hydrocarbons.

INTESTINAL GLYCOSIDASE DEFECTS

Hereditary diseases with insufficiency of intestinal lactase (78) and invertase (79) have been described. Oral lactose (78) or sucrose (79) tolerance tests fail to increase blood glucose, and lead to watery stools of low pH, containing lactic (79, 80) and other volatile acids (80) of microbial origin. The excellent state of health of a ten-year-old child with lactase insufficiency is in keeping with the endogenous metabolic formation of galactose for brain lipids (78). The normal response to maltose, in contrast to sucrose (80), would suggest that in man the major hydrolysis of these two disaccharides is by different enzymes. A similar conclusion was reached by fractionation studies of hog intestinal glycosidases (81). An unanswered question in the human studies is concerned with the fact that patients with invertase defects do not tolerate dextrin and show a decreased elevation in blood sugar following starch loads (80). The enzymes that achieve hydrolysis of the α -1,6 bond in the hog intestine [oligo-1,6-glucosidase (82), isomaltase (81)] were shown to be separable, by fractionation techniques, from either invertase (maltase I) which accounts for all of the sucrose and about one-third of the maltose hydrolyzing capacity (81), or maltases II and III, which account for the remaining two-thirds of the maltose hydrolyzing capacity. In the other direction, hog intestinal maltase was prepared essentially free of oligo-1,6-glucosidase activity (82). This evidence would indicate that either the specificity of human invertase is different from that of the hog or that in the

human there may exist an additional defect of α -1,6 link hydrolysis (83). The transient association of lactosuria and sucrosuria was reported (84, 85).

HETEROPOLYSACCHARIDES

Abnormal amounts of acid mucopolysaccharides in tissues of patients with gargoylism (Hurler's disease) have been reported (86, 87, 88). Chondroitin sulfate B and heparitin sulfate were recently identified (89, 90, 91). Excretion of large amounts of these two acid polysaccharides in urine was also observed (90, 91, 92) and a urine spot test with toluidine blue was devised (93). An abnormality of mucopolysaccharide metabolism (92), possibly in fibroblast differentiation (90), has been proposed.

In Marfan's syndrome, a decrease in serum seromucoid and an increase in serum acid polysaccharides were reported (94) with no alteration in the composition of the seromucoid. An increased urinary excretion of hydroxyproline in this disease was also observed (95).

AMINO ACIDS, PEPTIDES, AND PROTEINS

Argininosuccinic aciduria.—Argininosuccinic acid was identified as the unknown ninhydrin-reacting material on paper chromatograms of the urine of two siblings with mental retardation (96). The presence of higher concentrations in cerebrospinal fluid than in plasma was taken to indicate that argininosuccinic acid is synthesized in the central nervous system and then leaks into the blood (97). Like the other intracellular metabolites, ethanolamine phosphate and β -aminoisobutyric acid, it is excreted in the urine of humans without tubular reabsorption (98). Since argininosuccinic acid is an intermediate in urea biosynthesis in liver, it is perhaps surprising that affected patients had normal plasma urea concentrations and normal urine urea outputs, suggesting that the metabolic block occurs in brain but not in liver. In harmony with this idea is the demonstration of Sporn, Dingman, Defalco & Davies (99) that rat brain incorporates labelled arginine into urea *in vivo* and thus has the capacity to synthesize urea.

β -aminoisobutyric aciduria.— β -Aminoisobutyric acid was identified in some samples of human urine (100, 101) and a genetic basis for increased excretion established (102). Recent population studies by Blumberg & Gartler (103) revealed that over 80 per cent of Micronesians of the Marshall Islands who were examined were high excretors (highest percentage for the population groups thus far studied)—suggesting an origin in Asia for the high-excretor gene. It is agreed that β -aminoisobutyric acid is filtered in the kidney and excreted without tubular reabsorption (98, 104). Gartler (106) showed that, following administration of thymine, excretion of β -aminoisobutyric acid by high and low excretors did not differ. A marked difference in the excretion of β -aminoisobutyric, after intravenous administration of a test load, suggested that the difference between high and low excretors lay in its metabolism rather than in whether its formation was from thymine or by

excretion (105). Increased excretion following nitrogen mustard administration (107), as well as after uranium irradiation (108), is consistent with a metabolic origin from thymine derived from DNA breakdown. The work of the Finks *et al.* (109) Canellakis (110), Caravaca & Grisolia (111) demonstrated the conversion of thymine to β -aminoisobutyric acid through reductive degradation. A possible route from valine by way of a transamination reaction of methylmalonylsemialdehyde with glutamate was suggested by Kupiecki & Coon (112). Sutton (113) cited evidence in favor of the thymine origin, but suggested that the evidence is meager. If the transaminase step were involved in β -aminoisobutyric acid degradation one might wonder whether it or a subsequent step in the degradation of methylmalonylsemialdehyde may be involved in a metabolic block.

Cystathioninuria.—Harris, Penrose & Thomas (114) described cystathioninuria in a mentally defective patient in whose family two other members were found to excrete this substance. Tissue studies revealed an in-

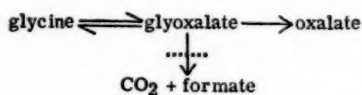


FIG. 1. Glyoxalate metabolism.

creased content of cystathionine in kidney and liver. Because of the presence normally of cystathionine in human brain (115) it was not possible to ascertain whether or not the amount present in brain was increased. A metabolic block at cystathionine cleavage, in the conversion of methionine to cysteine, was postulated (114).

Primary hyperoxaluria and oxalosis.—Studies of oxalate metabolism in man by tracer techniques showed that glycine is a significant precursor (116, 117). Oxalate was not appreciably further oxidized to CO_2 and was excreted almost quantitatively in urine (116). Recent studies focussed attention on glycine metabolism in this disease. In the face of a considerably increased incorporation of labelled glycine into oxalate in hyperoxalurics, the same proportion of urinary oxalate was derived from glycine (116, 117). Overproduction of oxalate from glycine and other precursors was therefore best explained by a block of glyoxylate degradation (116, 117), rather than by excess formation of glyoxalate. Enzyme sites considered include glyoxylic glutamic transaminase (118) and glyoxylic dehydrogenase (119). Improvement was noted on administration of benzoic acid, which led to an increased excretion of glycine as hippurate (see Figure 1). This disease points up the fact that only a small part of the total metabolism of a compound such as glycine need be involved to lead to the excessive formation of oxalate and the resultant disease state (117).

Maple-sugar-urine disease.—Menkes, Hurst & Craig (120) first reported the familial incidence of a fatal disease associated with the presence of an

organic acid in urine which smelled like maple sugar. A large increase of the branched chain amino acids leucine, isoleucine, and valine (121, 122) was found in urine and plasma (123). Examination of a urine specimen which was stored frozen for a year before death revealed increased amounts of the α -keto derivatives of these amino acids (123). Evidence indicates a block at a point beyond transamination (A) and either at or beyond oxidative decarboxylation (C) (Figure 2). Transaminase was demonstrated to be present in liver, brain, heart, and kidney obtained at autopsy (124). A moderate hypoglycemia which does not appear to be severe enough to explain the mental state, remains unexplained. The possible relationship to leucine-precipitated hypoglycemia (125) was mentioned.

Hypophosphatasia.—Phosphoethanolamine (126, 127), originally noted

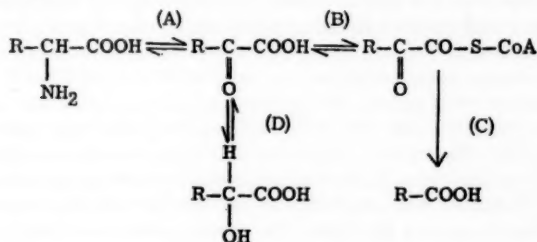


FIG. 2. Amino acid metabolism in maple-sugar-urine disease.

on paper chromatograms of urine, was identified chemically (128). The increased excretion of this substance appears to be related biochemically to the disease, and was used by Harris & Robson (129) for the detection of carriers. A genetic study indicated that 58 per cent of presumed heterozygotes were detected by urine chromatograms. The practical importance of increased phosphoethanolamine excretion is clearly evident from these studies.

PURINES AND PYRIMIDINES

Primary gout.—Evidence for increased urate generation from labelled precursors, as well as a decrease in the renal excretion of urate in primary gout, was discussed by Wyngaarden (38). After careful evaluation of the published data, he concluded that both factors are involved in generation of the hyperuricemia. Such a conclusion draws support from studies on xanthinuria, where the absence of xanthine oxidase is associated in the disease state with a defective tubular reabsorption of xanthine (130) [see following section]. In an approach to defining the site of the metabolic lesion, Wyngaarden, Jones & Ashton (131) demonstrated an increased specific activity of the ribose moiety of imidazole acetic acid riboside after ^{14}C -glucose administration to certain gouty subjects. An increased turnover of phosphoribosyl

pyrophosphate was deduced in keeping with previous evidence which suggested a failure of control at one of the early steps of purine biosynthesis (132). Since Gutman, Yu & Berger (133) provided evidence for renal tubular secretion of urate in man, the question of the nature of the renal defect was reopened. Adler & Gutman (134) reported the presence of 5-ribosyl uracil in urine of nongouty and gouty patients. This component was previously described in yeast RNA (135).

Xanthinosis.—Dickinson & Smellie (130) investigated a case of xanthinosis which was studied previously by Dent & Philpot (136). Using enzymic techniques, no uric acid was detected in plasma and at most only traces in urine. Plasma and urine contained elevated concentrations of xanthine and hypoxanthine, with xanthine predominating. The authors concluded that these findings were consonant with a virtual absence of xanthine oxidase. In addition, a high renal clearance of xanthine, but not of urate, was demonstrated, denoting a specific defect in tubular reabsorption. This important study delineates a metabolic as well as a renal defect in this disease.

Oroticaciduria.—The excretion of orotic acid in the urine of a child with a refractory megaloblastic anemia was described (137) and subsequently discussed (138). The anemia responded to feeding of a mixture of cytidylic and uridylic nucleotides. A block in pyrimidine metabolism subsequent to orotic acid formation was postulated. In abstract form (139) it was reported that erythrocyte assays for orotidylic pyrophosphorylase and orotidylic decarboxylase of the parents and two siblings indicated that both enzymes were defective. No defect was noted in a third sibling. Levels of aspartate carbamyl transferase and dihydroorotase were normal.

KIDNEY AND TRANSPORT

Just as the genetic diseases of metabolism provided an insight into cell metabolism, so a group of diseases has accumulated which centers chiefly about the kidney. The common factor in these diseases is an altered capacity to transport metabolites into or out of the kidney tubules and other cells. These diseases provide an insight into transport. Two further examples, xanthinuria and the trapping defect of hypothyroidism, are discussed in this review.

Cystinuria.—The work of Dent & Rose (140) and Stein (141) demonstrated that the coexcretion of cystine, lysine, arginine, and ornithine by cystinurics results from a defect in the reabsorption of these four amino acids by the kidney tubule. The genetics was elucidated by Harris and co-workers (142). A number of important studies have amply documented the renal tubular site (143, 144). Reviews were provided by Knox (145) and Harris (10).

Cystinosis.—Cusworth & Dent (98) showed with ion-exchange techniques that the aminoacidurias in the childhood Fanconi syndrome with cystinosis, as well as the adult Fanconi syndrome, were of renal tubular origin. In the adult disease, renal clearances of the amino acids were generally raised, and plasma levels were normal with the exception of glycine and proline, which

were increased. In the childhood disease, the amino acid excretion pattern was similar to that of the adult, but less marked. Renal clearances were increased plasma amino acid concentrations were not elevated [see also (145A)]. Clay, Darmady & Hawkins (146) elegantly demonstrated by dissection studies an altered nephron structure in this disease.

Hartnup's disease.—Milne, Crawford, Girao & Loughridge reported on their thorough studies (147). This disease, the features of which were previously described (148, 149), appears to be inherited in a recessive manner. After oral loads of L-tryptophan, kynurenine excretion was reduced in patients with the disease, as compared to controls. No formylkynurenine was detected. Excretion of indolic compounds in urine was increased and delayed after L-tryptophan loads. Tryptophan was detected in stools of patients with the disease, but not in stools of controls. A defect in gastrointestinal absorption of L-tryptophan was indicated by these studies. Increased excretion of indolic compounds was explained by a prolonged excretion of bacterial breakdown products from unabsorbed tryptophan. After DL-tryptophan loads, an increased tryptophan excretion in the urine of controls was detected—presumably the poorly metabolized D-isomer. In spite of higher initial concentrations in the urine of patients with the disease, only a slight increase in excretion was detected, again in keeping with a defect in gastrointestinal absorption. The pellagra-like rash would appear to be related to a defect in conversion of tryptophan to nicotinamide. Decreased conversion to kynurenine may be attributable to a defect in transport of tryptophan in liver. Nemeth & Nachmias (150) raised the question of a defect in tryptophan pyrrolase. Cusworth & Dent (98) demonstrated increased renal clearances for serine, glutamine/asparagine, histidine, threonine, glycine, and cystine. Tryptophan was not reported because of destruction on the column. Plasma levels of serine, threonine, and glutamine/asparagine were below normal in this study, and concentrations of proline and lysine were reported to be slightly increased. It would appear that the disease is marked by gross defects in absorption of amino acids in kidney, intestine, and possibly liver (147).

Glycinuria.—DeVries, Kochwa, Lazebnik, Frank & Djaldetti (151) reported glycinuria of a hereditary and presumably dominant nature in four members (three generations) of a family. Three had kidney stones. On analysis, oxalate, together with some glycine, was detected in one stone. Analysis of urine failed to detect elevated oxalate or the presence of glyoxalate, indicating that the oxalate of the stone probably did not arise from increased conversion from glycine. Plasma glycine was not elevated, and clearance was markedly increased, indicating a specific tubular reabsorption defect.

ENDOCRINES

Thyroid.—The incisive biochemical dissection of hypothyroid diseases was reviewed by Stanbury (152, 153) and McGirr (154). Five defects were established. In addition to defects in iodotyrosyl coupling (155), deiodination

(156, 157), conversion of iodide to organic form (158), a defect in iodine trapping or concentration was described (159). In the remaining group an abnormal serum iodoprotein is present, as evidenced by decreased extractability into acid butanol (160, 161).

A patient lacking the iodide-concentrating mechanism in thyroid was unable to concentrate iodide in saliva or gastric juice (159). Thyroid slices did not concentrate iodide. The gland contained a small amount of protein-bound iodine which apparently entered by diffusion. It was reported that treatment with an increased dose of KI led to improvement, the defect being overcome by an elevated concentration of blood iodide (152).

Haddad & Sidbury (158) directly demonstrated a defect in conversion of iodide to organic form. Monoiodotyrosine could not be detected following proteolytic digestion, or after incubation of particulate thyroid fractions with ^{131}I iodine and iodide with or without an H_2O_2 generating system. Previous studies showed that this defect was delineated by a large release of ^{131}I iodine from the thyroid after KSCN administration (153). This type was classified as a defect in peroxidase (154).

The dehalogenase defect was studied in detail by both direct and indirect methods, and is of considerable interest (154). Stanbury (152) reported that, in accord with theory, the defect was overcome by an increased dose of iodide. This may be an example of an enzyme defect concerned in modifying or controlling a biosynthetic sequence.

Adrenal.—Hydroxylation and other defects in the adrenogenital syndrome were reviewed by Wilkins (162), and Tomkins & McGuire (163). Bongiovanni (164) reported in preliminary form on a new lethal type in which a group of 3- β -hydroxy- Δ^4 steroids predominated with a deficiency of 3- β -hydroxy dehydrogenase. Prader & Siebenmann (165) described an adrenal lipid hyperplasia with cholesterol, but with no ketosteroids.

Diabetes.—The genetic aspects of human diabetes mellitus were reviewed by Steinberg (166), who concluded that the tendency toward diabetes was inherited in a recessive manner. Fajans & Conn (167) reviewed the work on latent diabetes detection with the cortisone-glucose tolerance test. A higher proportion of positive reactors among the nondiabetic relatives of diabetic patients than among controls was reported. In addition to the well known juvenile-onset and the maturity-onset types, a third category of lipotrophic diabetes was recognized (168). This disease was originally described by Lawrence (169); the subject was reviewed by Craig & Miller (170) and Schwartz, Schafer & Renold (171). "Insulin-like" activity was observed in plasma from patients with this disease. Pituitary hyperfunction or a failure of fat tissue to respond to insulin were discussed as possibilities. Louis, Minick & Conn (172) reported in abstract form that the disease may be related to an increased amount or activity of adipokinin. Chalmers, Pawan & Kekwick (173) had previously described an isolation procedure for the peptide. Other examples of insulin-resistant diabetes with demonstrable plasma "insulin-like" activity were described (174, 175).

The structure analysis of human insulin has now revealed a new site of amino acid substitution. Sanger (176) reports that the C-terminal residue of the B chain which is alanine in other species examined, is serine in the rabbit and threonine in man.

MUSCLE

Metabolism.—The severe limitation of muscle function associated with the phosphorylase defect of McArdle's disease was mentioned. The myoglobinuria which may be present suggests an alteration of membrane permeability. Other structural alterations in muscle were noted histologically (177). Thus, structural alterations appear coincident with an inability to degrade glycogen through phosphorylase. In Type II, cardiac or generalized glycogenosis, glycogen is present in large amounts in heart in a vacuolar form which tends to displace or destroy muscle fibers (179). A generalized amylo-1,6-glucosidase defect associated with myopathy was reported (31).

Shy & Magee (178) reported a new myopathy which was familial and nonprogressive in nature. Histologically, muscle fibers contained one or more longitudinal, somewhat centrally located areas which stained abnormally ("central core" disease). Dubowitz & Everson Pearse (180) studied muscle with "central core" disease by histochemical techniques. Central areas contained no glycolytic or oxidative enzymes. Remaining portions of the fibers were reactive and the presence of the enzymes was indicated. This unusual pattern differed markedly from muscle obtained from unaffected parents. The absence of enzyme activity in the central core may reflect a nonfunctional component whose chemical nature was not identified. Reference was made to similar abnormal findings in some cases of myotonia congenita. These studies would tend to indicate a rather extensive developmental defect in this disease.

Muscular dystrophy.—Dreyfus & Schapira (14) reviewed the biochemical and enzyme studies in muscle disease. The variable pattern of enzyme alteration in the diseased muscle was pointed out. Based on the work of Sibley & Lehninger (181), the diagnostic importance of the increased serum enzymes has become evident. Increased serum aldolase with muscle aldolase specificity was demonstrated in the myopathies, while increased serum aldolase, with liver aldolase specificity, was demonstrated in hepatitis or the liver glycogenoses (182). Ebashi, Toyokura, Momoi & Sugita (183) demonstrated increased serum creatine phosphokinase activity in muscular dystrophy [also noted by Schapira, Dreyfus, Schapira & Demos (184)]. The latter authors (184) studied 55 normal mothers with children who had the Duchenne type of myopathy. In fourteen, increased plasma aldolase was detected; in twelve, creatine phosphokinase was increased; while in eight, both enzymes were elevated. Chung, Morton & Peters (185) reported that heterozygous carriers of chicken muscular dystrophy had increased serum aldolase. Increased serum aldolase was also found in a minority of human carriers of the disease.

Increased turnover of amino acids in the protein (186, 187, 188) as well as increased incorporation of labelled glycine into nucleic acid (188) of dystrophic mouse muscle has been reported. These studies agree well with the increased number of cell nuclei observed histologically in diseased muscle, as originally reported by Michelson, Russell & Harman (189). Bourne & Golarz suggested that the disease may be one of connective tissue (190). Demos & Ecoiffier (191) noted circulatory abnormalities which may be significant in explaining the increased serum enzyme levels.

DRUG SENSITIVITY

Cholinesterase.—Lehmann & Ryan (192), Allott & Thompson (193) and Kalow (194) reported a familial incidence of decreased serum pseudo-cholinesterase. Kalow & Lindsay (195) reported that the serum enzyme of such patients apparently differed from that of normals, as determined by substrate affinities as well as by lessened sensitivity to inhibitors. The decreased sensitivity to inhibition by dibucaine (nupercaine, percaïne) was standardized (196). Sera obtained from about 1700 students, laborers, and hospital patients were studied and fell into three groups (197). The data best fit the model of two autosomal allelic genes without dominance, yielding normal, atypical, and mixtures of the two enzymes. Some deviations from this model were noted. Harris & Whittaker (198) found that an inhibitor extracted from potato peel sharply distinguished the three groups and correlated well with the dibucaine inhibitions.

Kalow & Davies (199) studied sixteen cholinesterase inhibitors. Differences in susceptibility to inhibition were not observed with tetraethyl pyrophosphate and di-isopropyl fluorophosphate, but the atypical enzyme had decreased sensitivity to all other inhibitors tested. Since tetraethyl pyrophosphate and di-isopropyl fluorophosphate react with the esteratic site of the enzyme (200), this suggests that the esteratic sites of typical and atypical cholinesterases may be identical. Inhibition studies with decamethonium and succinylcholine indicate that the difference at the anionic site could be because of a relatively weak effective charge in the atypical enzyme (199). Marton & Kalow (201) succeeded in separating normal serum cholinesterase from aromatic or A esterase by paper electrophoresis. These series of important studies have focused attention on the genetic control of the synthesis of an altered enzyme.

Primaquine sensitivity.—Beutler (202, 203) reviewed the glucose-6-P dehydrogenase defect in drug- and fava-bean-induced hemolytic anemia. Carson (204) brought together the several differences from normal which characterize the sensitive erythrocyte. Recent findings include decreased incorporation of ^{14}C -glycine into glutathione (205)—possibly related to a decreased glutathione content. Increased levels of NADP, and possibly of NAD were noted (206), in keeping with decreased glucose-6-P dehydrogenase (207) and increased glutathione reductase (208). Interestingly, an increase of aldolase, but not of glyceraldehyde-3-P dehydrogenase, was re-

ported (209). It is apparently not clear whether the increases in these two enzyme activities are attributable to assay of younger erythrocytes, or represent compensatory mechanisms (204). Catalase was shown to be deficient by Tarlov & Kellermeyer (210), while Tarlov [see (204)] found that total lipids were decreased, and that glucose utilization in the presence of methylene blue was decreased. The multiple facets of a defect in the pentose phosphate pathway are evident from these studies.

Decreased glucose-6-P dehydrogenase activity was demonstrated in lens by Zinkham (211), in leucocytes of affected Caucasians by Marks & Gross (212), but not in leucocytes or liver of affected Negroes (213). Kirkman (214) and Marks (213) summarized studies with partially purified enzyme preparations, demonstrating no detectable difference between normal and sensitive erythrocyte glucose-6-P dehydrogenase. Of considerable interest is the fact that Kirkman, Riley & Crowell (215) reported altered enzyme constants in a purified erythrocyte glucose-6-P dehydrogenase from an infant with a related disease, nonspherocytic hemolytic anemia.

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BIOCHEMISTRY OF FUNGI^{1,2,3}

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INTRODUCTION

The number of compounds isolated from molds continues to increase rapidly. The *List of Fungal Products*, published by Shibata, Natori & Udagawa is an invaluable guide to the literature; it contains 565 references to work published up to about the end of 1960 (1). This review will outline some of the mechanisms observed for the biosynthesis of mold metabolites. In view of the volume of work and the existence of other reviews, penicillin (2, 3), the carotenoids (4), and riboflavin (5) will not be considered here.

The extensive application of tracer techniques has revealed a fundamental role for many of the familiar small molecules such as formate, acetate, propionate, malonate, etc. In some cases, biosynthetic mechanisms have been considered as so well established that structural assignments have rested on biogenetic considerations. A recent example is echinulin (6). A difficulty confronting the reviewer is that extensive schemes are frequently based on a single experiment with a precursor such as acetate, and often at a low level of incorporation. In general, the added precursor is not used as the sole carbon source; the sequences between, say, sugar and acetate are usually ignored. Further, there is usually little or no evidence for postulated precursors, and the confirmation of biogenetic schemes by direct enzymatic experiments is a rarity. Many admittedly attractive schemes are based only on isolation of postulated precursors from a variety of organisms under an assortment of conditions. Although many general outlines are emerging in this field, solid documentation is badly needed.

Mold metabolites are frequently formed in high yields, often under conditions of high sugar concentration and low pH. They have been termed

¹ The survey of the literature for this review was concluded in October 1961. The review has included work on the actinomycetes as well as the true fungi.

² The following abbreviations are used: CoA for coenzyme A; Me for the group, CH₃; Et for the group, C₂H₅. The following symbols for labeled atoms have the same significance throughout the manuscript; other symbols used less frequently are defined for each Figure in which they appear. ■ = carbon from methyl of acetate; ● = carbon from carboxyl of acetate; □ = carbon from either methyl of acetate or methylene of malonate; ○ = carbon from either carboxyl of acetate or malonate; ○ = carbon from carbon dioxide; ▲ = carbon from a C₁ unit such as formate or the methyl of methionine; ▽ = carbon from the 2 position of mevalonic acid; ♦ = carbon from the carboxyl of propionate.

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"shunt" or "overflow" metabolites (7). One general conclusion, resulting from enzymatic studies, is that overflow metabolites may accumulate either because of the absence of an enzyme in a fundamental pathway, or because of the acquisition of a new enzyme that is unique to a particular process. Examples are the loss of isocitric dehydrogenase activity during accumulation of citric acid by *Aspergillus niger* (8) and the acquisition of cis-aconitic decarboxylase in itaconic acid formation in *A. terreus* (9). Another reason for accumulation of shunt metabolites is the absence of a nutritional factor or factors required for the "normal" pathways of glucose dissimilation. This is the case for the accumulation of polyacetylenes in the Basidiomycete, B 841 (10). It is also of general interest that the benzene ring may be cleaved in formation of other metabolites, e.g., gentisaldehyde \rightarrow patulin; orsellinic acid \rightarrow penicillic acid.

Acetic acid has an important role in the formation of shunt metabolites. Birch & Donovan in 1953 (11, 12) and Robinson in 1955 (13) outlined theories of "head-to-tail" acetate condensations to account for the biosynthesis of many mold compounds. The incorporation of labeled acetate into citric acid (14), itaconic acid (15), and kojic acid (16) had been previously demonstrated, while in *Neurospora crassa* the head-to-tail condensation of acetate to supposed C_4 precursors of isoleucine and valine had been described, as such, as early as 1950-1951 by Tatum & Adelberg (17). The synthesis of a key aromatic compound, 6-methylsalicylic acid, from acetate-1- C^{14} with the predicted distribution from head-to-tail condensations was shown for *Penicillium griseo-fulvum* by Birch, Massy-Westropp & Moye in 1955 (18).

Although considerable evidence (to be discussed subsequently) has supported the validity of the head-to-tail polyacetic condensations, recent developments in connection with the role of malonate in fatty acid biosynthesis suggested that a modification of the acetate hypothesis might be necessary in some cases. Lynen, in 1959, speculated that malonyl CoA might be the real condensing agent in the "polyacetic" series, and as an example derived the sixteen carbon atoms of the plant-product eleutherinol from eight malonate units (19). Direct tracer evidence was obtained by Bentley & Keil in 1961, showing that both acetate and malonate units in a one-to-three ratio are required for the synthesis of orsellinic acid, and hence of penicillic acid (20). A general equation analogous to that for fatty acid synthesis may, therefore, be written:



It is now clear that in many cases, "overflow" metabolism is a deviation from the normal pathways of lipid synthesis. Although the utilization of added C^{14} -labeled malonic acid apparently occurs very readily in some organisms, it should be noted that in *P. chrysogenum* Olson & Chain found little incorporation of the free acid into mycelial components; a significant incorporation was observed, however, with the ethyl ester (21).

A requirement for both acetate and malonate has been shown subse-

quently for the biosynthesis of several other mold metabolites, including the important compound, 6-methylsalicylic acid (22, 23). An enzyme extract, synthesizing radioactive 6-methylsalicylate from acetyl-1- C^{14} CoA only in the simultaneous presence of malonyl CoA and reduced triphosphopyridine nucleotide has been obtained from *P. patulum* by Lynen & Tada (24).

Although it seems likely that many more compounds are formed by the condensation of 1 acetyl CoA and n malonyl CoA units, the term "polyacetic" will be used here for convenience. Further, acetate and malonate will

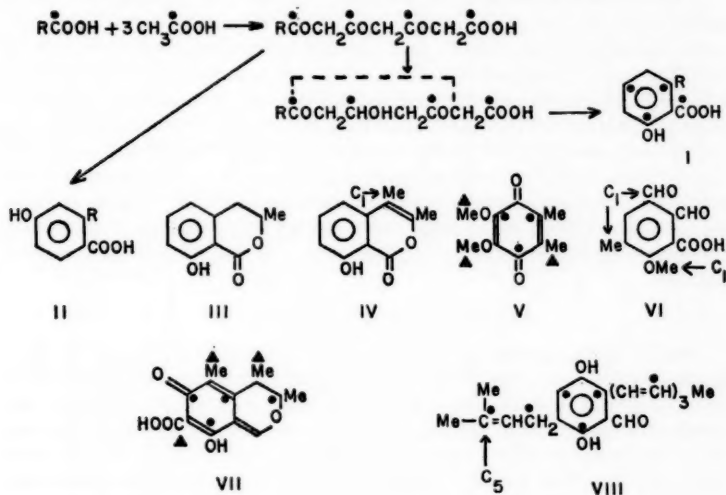


Fig. 1. Derivatives of phenol formed by the polyacetic pathway.

be used without qualification as to the precise reactive species: presumably in all cases, these molecules react as the CoA derivative.

With the exception of mevalonic acid, there is little evidence about possible intermediates between acetate and malonate and the final metabolite. The polyketomethylene intermediates (e.g., Fig. 1) are written for ease of understanding but have never been detected. From studies of the formation of anthraquinones and diantraquinones in *P. islandicum*, Ehrensward & Gatenbeck have concluded that independent routes lead separately to each of several products and that no definite chemical entities exist as intermediates (25). The different end products are regarded as "statistical variations" from the same condensation process proceeding through the same enzyme complex. Similarly, Lynen & Tada have suggested a multi-enzyme complex for the biosynthesis of 6-methylsalicylic acid, similar to that proposed for fatty acid synthesis (24). On the other hand, the facile chemical

cyclization of the O-dimethyl derivative of curvularin to a naphthol has led to the suggestion that such macrocyclic lactones may be intermediates in the biosynthesis of polycyclic aromatic compounds (26). The chemical formation of aromatic compounds from β -polyketones has been studied by Birch *et al.* (27). In a few cases, the utilization of a preformed aromatic structure for synthesis of a subsequent molecule, without cleavage of the aromatic ring, has been established (see utilization of 6-methylsalicylic acid, Table I, and orsellinic acid, Table II).

Further modifications of a basic unit derived from acetate are always possible by addition of OH groups or halogens, by addition of other carbon units, which are frequently C_1 or $(C_2)_n$, and by modification of the CH_3 or $COOH$ groups introduced by acetate itself. In the latter case, the oxidations $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow COOH$ are well known; less common are the reductions of $COOH$ to CHO , CH_2OH , and CH_3 . Formation of aromatic aldehydes and alcohols from acids in molds is well documented (28, 29) and a CH_2OH derived from $COOH$ is found in the cyclohexanone ring of palitantin (Table II). The biosynthesis of threonine from oxaloacetic acid in *N. crassa* requires the reduction $COOH \rightarrow CH_3$ (30), and a methyl group derived from acetate carboxyl occurs in mycelianamide (see page 618) and possibly in javanicin (25, 31).

POLYACETIC AROMATIC COMPOUNDS

The simple aromatics are numerous and well studied; they will be summarized with the aid of tables and figures, with textual comment reserved for items of special interest. These aromatics may be classified in part by the apparent retention of one, two, or three of the original oxygen functions. They will be described here as derivatives of phenol (one OH) or resorcinol (two OH) produced by formal aldol-type condensations, or as acylphenols with one, two, or three OH groups produced by formal C-acylation reactions. The few benzenoid compounds without any OH groups are generally derivatives of cinnamic acid, and are more likely formed by the shikimic acid pathway than by the polyacetic pathway. A unique derivative of benzoic acid is the recently isolated *p*-methylnitrosoaminobenzaldehyde from surface cultures of *Clitocybe suaveolens* (32).

Phenol derivatives.—These compounds are outlined in Table I and Figure 1. The simplest compound, 6-methylsalicylic acid, I, $R = Me$, has been extensively studied and its involvement in patulin biosynthesis will be discussed later. It has been reported that 6-methylsalicylic acid is converted to 2-methoxy-5-methylbenzoquinone in *Lentinus degener* and to aurantioglucladin, V, in *Gliocladium roseum* without breakdown to acetate (40). Although most of the monohydric phenols are derivatives of ortho-hydroxybenzoic acid, para-hydroxybenzoic acids, II, are theoretically possible, Citrinin, VII, may be derived from such a compound.

Resorcinol compounds.—These derivatives are shown in Table II and Figure 2. Although the parent of this series, orsellinic acid, is a common

TABLE I*
COMPOUNDS RELATED TO PHENOL DERIVED BY THE POLYACETIC PATHWAY

No.	Name	Organism	Added units	Tracers	Reference
I, R=Me	6-Methylsalicylic acid	<i>P. griseofulvum</i> , <i>P. urticae</i> , <i>P. patulum</i>		Ac-1, Mal-2	18, 22, 23, 24, 33, 34
I, R=CHO	2-OH-6-CHO-Benzoinic acid	<i>P. patulum</i>			34
I, R=COOH	3-OH-Phthalic acid	<i>P. islandicum</i> , <i>P. patulum</i>		Ac-1	34, 35, 36
III	Mellein (ochracin)	<i>A. ochraceous</i> , <i>A. melleus</i>			37
IV	Oospolactone	<i>Oospora</i> sp.	C-Me		38, 39
	2-MeO-5-Me-Benzoinquinone	<i>Lentius degener</i>	2 OH, O-Me	Ac-1, Ac-2, 6-MS	40
V	Aurantio-gladiolin	<i>Gladiolium roseum</i>	3 OH, 2 O-Me, C-Me	Ac-1, 6-MS, C ₁	40
VI	Gladiolic acid	<i>P. gladioli</i>	O-Me, C-Me, CHO		41
VII	Citrinin	<i>P. citrinum</i>	2 C-Me, COOH	Ac-1, C ₁	42, 43
VIII	Auroglaucon	<i>A. novus</i>	OH, C ₆	Ac-1, MVA-2	44

* In this and the following tables, the following abbreviations have been used for the various isotopic substrates: acetate-1-C¹⁴ = Ac-1; acetate-2-C¹⁴ = Ac-2; malonate-2-C¹⁴ = Mal-2; compounds such as formate or methionine giving rise to a C₁ unit = C₁; mevalonate-2-C¹⁴ = MVA-2; 6-methylsalicylic acid, biosynthesized from acetate-1-C¹⁴ = 6-MS; orsellinate-5-C¹⁴ = Or-5.

TABLE II
COMPOUNDS RELATED TO RESORCINOL DERIVED BY THE POLYACETIC PATHWAY

No.	R	R'	Name	Organism	Added units	Tracer	Reference
IX	Me	H	Orsellinic acid	<i>Chaetomium cochliodes</i> P. <i>barnense</i> , etc.		Ac 1, Mal-2	46, 47, 48
IX	COOH	H	3,5-(OH) ₂ -Phthalate	<i>P. breviscompactum</i>			49
IX	CHOHCOMe	H	"C ₁₀ Acids"	<i>P. breviscompactum</i>			50
IX	CHOHCOMe	OMe	Ustic acid	<i>A. ustus</i>	OH, O-Me		54
X			Palitantin	<i>P. cyclopium</i>	OH	Ac-1	52, 53
XI			Cyclopaldic acid	<i>P. cyclopium</i>	C-Me, O-Me, CHO	Ac-1, C ₁	53
XII	H	OH	Flavipin (R'' = Me)	<i>A. flavipes</i> , etc.	C-Me, OH		55
XII	Me	Me	Quadrilineatin (R'' = H)	<i>A. quadrilineatus</i>	C-Me, O-Me		56
XIII			Sclerotiorin	<i>P. multicolor</i>	3 C-Me, Cl, OAc	Ac-1, Ac-2, C ₁	43, 57
XIV	CH:CHMe	C ₆ H ₁₁	Rubropunctatin	<i>Monascus rubropunctatus</i>	C-Me, OH		58
XV			Mycophenolic acid	<i>P. breviscompactum</i>	C-Me, O-Me, 2 C ₆	Ac-1, MVA-2, C ₁ , Or-5	60, 61, 62

component of lichen acids, it was not identified in a pure mold culture until Mosbach's work in 1959 (45). In the same year, Gatenbeck & Mosbach carried out an important experiment with acetate-1- O^{18} , showing a surprisingly large incorporation of O^{18} into the metabolite and little exchange with water of the medium (46). The O^{18} content of the COOH group was half that of the OH groups; this would be predicted if a CoA derivative were involved, with hydrolysis of the terminal thioester introducing O^{18} from the medium. This is one of only two cases where retention of oxygen in a polyacetic compound has been experimentally determined with O^{18} -labeled acetate. Three " C_{10} acids" (IX, $R = CH_2COCH_3$, or $CHOHCOCH_3$, or $COCOCH_3$) from *P. brevi-compactum* (50) are probably acetate-derived. Godin has investigated

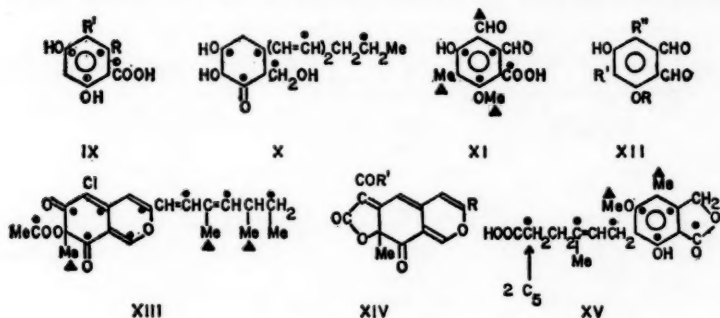


FIG. 2. Derivatives of resorcinol formed by the polyacetic pathway.

the amounts of these acids, and tricarboxylic acid cycle acids, present under a variety of conditions (51). Although palitantin is not strictly aromatic, it is included for convenience. The polyacetic pathway for palitantin requires the reduction $COOH \rightarrow CH_2OH$; the CH_2OH is not derived from mevalonate under conditions where the latter is used for steroid synthesis (52). Compounds of the general structure, XIV, such as rubropunctatin, rotiorin, and monascorubrin (58, 59) are apparently derived similarly to sclerotiorin, but with the addition of an acetate-derived chain. They are exemplified in Table II by rubropunctatin.

In mycophenolic acid, a C_5 unit is derived from mevalonate. In early experiments with acetate-1- C^{14} present over a 14-day period, the degree of labeling was the same in the ring and in the mevalonate-derived side chain (61). In further experiments, marked changes in the C^{14} content of the side-chain atoms relative to those of the ring have been observed that are dependent on the time of incubation with the labeled acetate (62). It is of interest that, although orsellinic acid-5- C^{14} was only poorly utilized for mycophenolic acid biosynthesis, the observed activity was predominantly at C_6 , as expected (62).

Thomas has postulated that a number of more complex resorcinol-related products may be derived from "orsellinyl triacetic acid," XVI (31). Many different condensations of this compound are theoretically possible: those indicated in Figure 3 are consistent with the observed isotope distributions in the following compounds: citromycetin, XVII, from *P. frequentans* (43); alternariol, XVIII, $R = H$, and its monomethyl ether, XVIII, $R = Me$, from *Alternaria tenuis* (31, 63); griseofulvin, XX, from *P. griseo-fulvum* (22, 64, 65). Other compounds derivable from XVI include fulvic acid by a 3-2' OH condensation (31, 66); purpurogenone, fusarubin, and javanicin by a 3-8 condensation (31, 67); rubrofusarin by a 1-8 condensation (68, 69); the benzophenone, XIX, $R = R' = R'' = R''' = H$, by a 1-6 condensation (31). The latter compound is the likely precursor for griseofulvin; and the sequence, griseo-

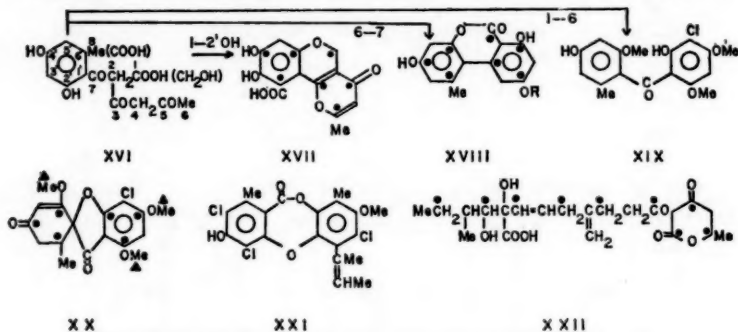


FIG. 3. Products formally derived from orsellinyl triacetic acid.

phenone C, XIX, $R = R'' = H$, $R' = R''' = Me$, \rightarrow griseophenone B, XIX, $R = Cl$, $R'' = H$, $R' = R''' = Me$, \rightarrow griseophenone A, XIX, $R = Cl$, $R' = R'' = R''' = Me$, has been established (70). Dehydrogriseofulvin (71) is believed to be formed from griseophenone A by oxidation to a diradical, followed by phenol coupling (72). Subsequent reduction yields griseofulvin. The chemical syntheses of griseofulvin provide a laboratory analogy for these ideas (71, 73). Further evidence for the role of the benzophenones is the isolation of the corresponding griseoxanthone C, 1,6-dihydroxy-3-methoxy-8-methylxanthone (74), and its marked accumulation when chlorination of griseophenone is inhibited (70).

Another benzophenone, sulochrin (methyl ester of 2,6,4'-trihydroxy-2'-methoxy-4-methyl-6'-carboxybenzophenone) is also the likely precursor of dechlorogeodin (Nishikawa's compound E, a spirane), thence asteric acid (diphenyl ether), and finally dechlorogeodioxin (75, 76, 77). A parallel series of chlorine-containing compounds is 3,5-dichlorosulochrin, geodin, geodin hydrate, geodioxin (78). The parent benzophenone may be formally derived from orsellinyl triacetic acid with a subsequent C_2 addition and decarboxylation (31), or perhaps by a C_1 addition. An alternative is that sulochrin is an

oxidation product of the anthraquinone, emodin, XXIX, $R=OH$, $R'=R''=H$ (25). Roberts suggests that nidulin, XXI, is also acetate-derived, with two C-Me groups being introduced; the postulated reaction is a condensation of a C₆-C₁₀ unit (79). An alternative is derivation from a benzophenone unit with 3 C-Me groups added. It is possible that the chlorine atom and the isobutenyl group in formula XXI may be interchanged.

There is no information to distinguish between the postulated use of orsellinyl triacetic acid and straight-chain precursors in the cases where both are theoretically possible. The need for malonate, for example in the synthesis of orsellinic acid itself, suggests a straight-chain precursor. Thomas has pointed out that a branched-chain precursor of orsellinic acid is, in fact, the open-chain form of dehydroacetic acid (31). It is perhaps fortuitous that a

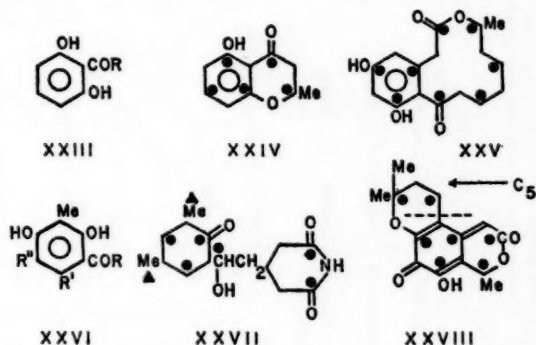


FIG. 4. Acylphenols and related compounds.

reduced derivative of dehydroacetic acid is alternaric acid, XXII, formed by *Alternaria solani* and shown by Turner (80) to be acetate- and formate-derived.

Acylphenols.—A condensation of polyacetic units, involving the terminal COOH, or other CO group, is possible, leading to acylphenols with up to three OH groups. Griseofulvin, XX, is actually a mixed resorcinol-acylphloroglucinol type. Other examples are shown in Table III and Figure 4.

Anthraquinones.—Evidence for the predicted isotope distribution, shown in Figure 5, has been obtained for emodin, XXIX, $R=OH$, $R'=R''=H$ (obtained from the dimer, skyrin), and islandicin, XXIX, $R''=OH$, $R=R'=H$, in *P. islandicum* (86, 87); in helminthosporin, XXIX, $R'=OH$, $R=R''=H$, from *Helminthosporium gramineum* (88); and in cynodontin, XXIX, $R'=R''=OH$, $R=H$, from *Phoma terrestris* (89). In all of these cases, eight acetate units have been condensed with loss of the terminal COOH. This group is apparently still retained in endocrocin, XXX, $R=OH$, $R'=R''=H$, and clavorubrin, XXX, R' or $R''=OH$, $R=H$, from *Claviceps*

TABLE III
ACYLPHENOLS DERIVED BY THE POLYACETIC PATHWAY

No.	R	R'	R''	Name	Organism	Added units	Tracer	Refer- ence
XXIII	Me			2,6-(OH) ₂ -Acetophenone	<i>Daldinia concentrica</i>			81
XXIII	MeCH ₂ CH ₃			2,6-(OH) ₂ -Butyrophenone	<i>Daldinia concentrica</i>			81
XXIV				5-OH-2-Me-Chromanone (+related chromone)	<i>Daldinia concentrica</i>		Ac-1	81
XXV				Curvularin	<i>Curvularia</i> sp., <i>P. steckii</i>		Ac-1	26, 82
XXVI	Me	H	Me	Clavatol	<i>A. clavatus</i>	2 C-Me		83
XXVI	(CH:CH) ₂ Me	H	Me	Sorbicillin	<i>P. notatum</i>	2 C-Me		84
XXVI	Me	OH	H	Usnic acid precursor	Lichen acid	C-Me		72
XXVII				Cycloheximide	<i>Streptomyces</i> sp.	2 C-Me+glu- tarate	Ac-1, C ₁	85
XXVIII				Fuscin	<i>Oidiodendron fuscum</i>	OH, C ₃	Ac-1, MVA-2	62

purpureae (90). The compounds islandicin and emodin also incorporate acetate-1- O^{18} , three O^{18} atoms being found in islandicin and four in emodin (87). Evidence suggesting that the anthraquinones and bianthraquinone compounds of *P. islandicum* are formed independently of each other has been presented by Gatenbeck (25, 91, 92).

Perinaphthhenones.—Atrovenetin, $C_{19}H_{18}O_6$, from *P. atrovenetum*, herqueinone, $C_{20}H_{20}O_7$, and norherqueinone, $C_{19}H_{18}O_7$, from *P. herquei* form a group of related perinaphthhenones whose structures are not wholly resolved. In particular, the position of attachment of a C_5 "reversed" isoprene unit is not definite (93). From *P. herquei* Thomas (93) isolated norherqueinone and degraded this to norxanthoherquein, XXXI, and methyl isopropyl ketone. With mevalonate-2- C^{14} the latter ketone contained all of the activity. The distribution pattern shown in Figure 5 was obtained from acetate-1- C^{14} .

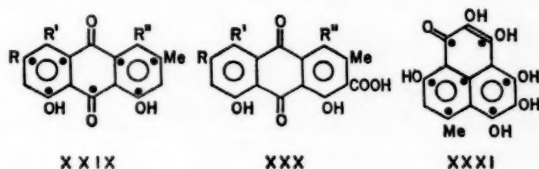


FIG. 5. Anthraquinones and perinaphthhenones.

COMPOUNDS RELATED TO THE POLYACETIC AROMATIC PATHWAY

Patulin.—Many other compounds, shown in Figure 6, have been isolated from cultures of *P. urticae* (*P. patulum*) producing patulin (33, 34, 94). Tracer experiments by Bu'Lock & Ryan (95) and Tanenbaum & Bassett (96) indicated a close relation between the synthesis of patulin and 6-methylsalicylic acid and were consistent with Birkinshaw's suggestion (97) that gentisaldehyde, XXXII, was a probable precursor of patulin, XXXIII, requiring an oxidative fission of the aromatic ring and a rearrangement. More recently, Bassett & Tanenbaum obtained cell-free enzyme preparations catalyzing the conversion of glucose, acetyl CoA, or 6-methylsalicylic acid to patulin (98). In experiments with labeled acetate or glucose, only traces of 6-methylsalicylic acid were detected. Furthermore, the addition of 6-methylsalicylic acid to the reaction mixture repressed the endogenous formation from acetyl CoA precursors. It is thus possible that a C_5 open-chain precursor yields patulin directly and also serves as a precursor for the other acetate-derived aromatics, as previously suggested by Ehrensward (33).

The role of gentisic acid is not clear; although some experiments have apparently shown net synthesis of patulin from gentisate (94), C^{14} -labeled gentisate was not converted to patulin in replacement experiments, while in tracer experiments with acetate-2- C^{14} , 6-methylsalicylic acid and patulin

were labeled but not gentisate (96). It is possible that gentisate is derived from a second pathway of aromatic synthesis, that via shikimic acid. This coexisting pathway is apparently used for the synthesis of the aromatic amino acids, pyrogallol, etc. (33, 94, 96). In replacement cultures, shikimic acid was converted to *p*-hydroxybenzoic acid, not to patulin (94). The present conclusions are summarized in Figure 6.

Tropolones.—Evidence that the seven-membered aromatic ring found in mold tropolones is derived by a modified polyacetic pathway has been presented by Bentley for stipitatic (XXXV, R = COOH) and stipitatic acids (XXXV, R = H) from *P. stipitatum* (99, 100) and by Richards & Ferretti for puberulonic (XXXVI, R = COOH) and puberulic (XXXVI, R = H) acids from *P. aurantio-virens* (101, 102). The "extra" atom of the tropolone ring is derived from formate. Furthermore, two carbon atoms (C₄–C₈) are derived

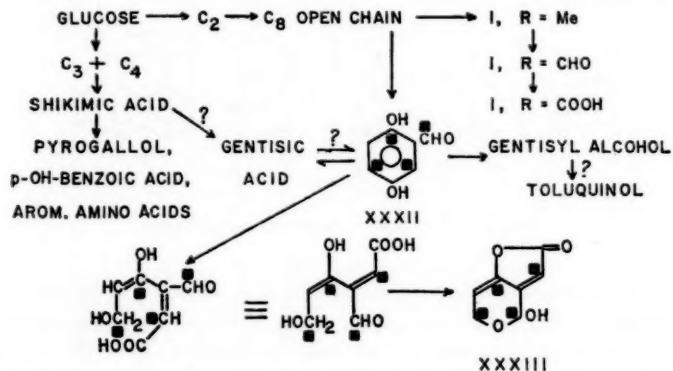


FIG. 6. Biosynthetic routes to aromatic compounds in organisms producing patulin.

more effectively from acetate than from malonate, indicative of a condensation of 1 acetyl CoA + 3 malonyl CoA (103, 104). It has been proposed (100) that orsellinic acid is a likely precursor of the tropolone ring as shown in Figure 7; however, orsellinic acid cannot be identified in the culture fluid of *P. stipitatum* nor is added C¹⁴ orsellinic acid significantly converted to tropolones by this organism (105). Supporting the theory is the fact that the labile carboxyl groups of stipitatic and puberulonic acids have a different origin. In the case of stipitatic acid, C₉ is derived from the carboxyl group of acetate (100). In puberulonic acid, C₉ is derived neither from acetate carbons nor, as predicted, from formate (100, 101, 102). Surprisingly, however, from formaldehyde and glycine-2-C¹⁴, C₉ contained respectively 8 and 14 per cent of the total activity (106), and from glucose-1-C¹⁴, 13 per cent of total activity (102). It seems to be established that the C₉ of puberulonic acid is derived from a C₁ unit but that added formate does not equilibrate with the

C₁ pool. It should also be pointed out that the postulated intermediate, XXXIV, is likely to be a precursor for cyclopolic and cyclopaldic acids, XI, and has the same labeling pattern as citrinin, VII.

A possible role for shikimic acid (12) in tropolone biosynthesis is ruled out by a comparison of the specific activities of stipitatic acid and mycelial phenylalanine and tyrosine derived from glucose-1-C¹⁴ (107), and by the failure of labeled shikimic acid to be converted to tropolones (105). A C₃ + C₃ condensation proposed by Levin & Racker (108) is not in accordance with the isotope data. Cell-free extracts carrying out the decarboxylation of the tropolone dicarboxylic acids have been prepared and partially purified by Bentley & Thiessen (109, 110). It is postulated that this decarboxylase enzyme is responsible for the *in vivo* accumulation of the monocarboxylic acids.

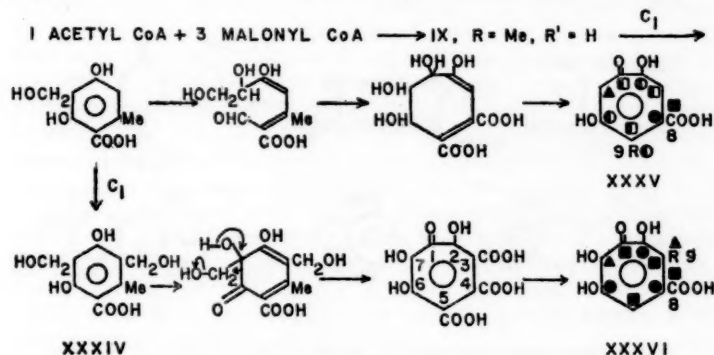


FIG. 7. Postulated mechanisms for the biosynthesis of tropolones. Two possible mechanisms for expansion of the six-membered ring are indicated. The scheme involving the open-chain aldehyde, leading to XXXV, is that of Bentley (100). That involving the pinacol-pinacolone rearrangement is that of Ferretti & Richards (102). In this scheme, a nuclear OH group is supposed to be introduced after the formation of the tropolone ring; this reaction has not been shown here as a separate step.

AROMATIC COMPOUNDS FROM SHIKIMIC ACID

The shikimic acid pathway seems to be used much less commonly for synthesis of aromatic overflow metabolites than the polyacetic pathway. The possible coexistence of the shikimic and polyacetic pathways has already been discussed in connection with patulin. Shikimic acid itself has been identified in 30-hour cultures of *P. griseo-fulvum* (111) and as its phosphate in *Lentinus lepideus* (112). In a triple mutant of *N. crassa* (requiring tryptophan, phenylalanine, and tyrosine for growth), large amounts of prephenic acid (up to 600 mg per liter) accumulate when the mold is grown at neutral pH with forced aeration (113). In the (arom) mutant of *N. crassa*, Tatum

phenoxazinone portion in *S. antibioticus*, but acetate is only a poor precursor of this moiety (127). The observed conversion of L-tryptophan to kynurenine, anthranilic acid, and hydroxyanthranilic acid by *P. notatum* is of interest in this connection (128). From methionine (C^4H_3), activity was observed in sarcosine, N-methylvaline, and the phenoxazinone portion (127). Similarly, in an unidentified *Streptomyces*, the two nuclear methyl groups of the actinomycin were specifically derived from methionine (129).

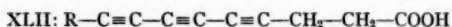
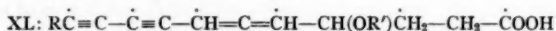
Preliminary evidence has been obtained by Luckner (130) for the biosynthesis of viridicatin, 2,3-dihydroxy-4-phenylquinoline, in *P. viridicatum* by condensation of anthranilic acid and a "phenyl propane" unit, probably derived by the shikimic acid pathway.

A role for β -keto adipic acid, possibly derived from aromatic compounds of the shikimic acid pathway, has been discovered in the biosynthesis of caldariomycin, 2,2-dichloro-1,3-cyclopentanediol, in *Caldariomyces fumago* (131, 132). A soluble, multicomponent, enzyme system, β -keto adipate chlorinase, converting β -keto adipic acid to δ -chlorolevulinic acid, has been studied in detail by Shaw & Hager (133, 134); the process is oxidative and requires the participation of H_2O_2 or of a glucose oxidase system to generate H_2O_2 . The chloroperoxidase component of the chlorinase system also converted 2-chloro-1,3-cyclopentanedione to 2,2-dichloro-1,3-cyclopentanedione. The latter compound is the probable immediate precursor of caldariomycin, via reduction. Although it appears likely that β -keto adipate is a precursor of caldariomycin, it is not clear whether the first cyclic product is cyclopentanedione, or the monochloro compound. Both Cl^{36} -labeled δ -chlorolevulinic acid and also Cl^{36} -2,2-dichloro-1,3-cyclopentanedione are converted to caldariomycin by *Caldariomyces fumago* cultures without hydrolysis to chloride ion (131, 132).

POLYACETYLENES

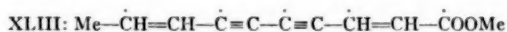
Many acetylene compounds are known in fungi. Using an unidentified Basidiomycete, B 841 (probably a *Poria* sp.), Bu'Lock & Leadbeater (10) observed that the highest yields of polyacetylenes were obtained in replacement cultures using 4 per cent glucose rather than the complete medium used for growth. Four per cent glucose plus corn-steep water (20 ml per liter) showed the same behavior as the complete medium. It was concluded that polyacetylene formation represented an alternative metabolic pathway for some product(s) from glucose dissimilation, the other metabolic routes for which show a partial requirement for factors in the corn-steep water. The principal metabolites of this organism are nemotinic acid, XL, $R=R'=H$, and odysic acid, XL, $R=Me$, $R'=H$. Acetate was well incorporated (15 to 20 per cent) into nemotinic acid with the utilization of six acetate units; odysic acid may have been a precursor for nemotinic acid after oxidation and decarboxylation (135). Decarboxylation of the acetylene compounds XLI and XLII ($R=COOH$) to, respectively, XLI and XLII ($R=H$) with

odd numbers of carbon atoms has been observed enzymatically with a cell-free extract from *Coprinus quadrifidus* (136).



From B 841, a xyloside derivative of nemotinic acid, XL, R = H, R' = β -D-xylosyl, has also been isolated (137). When glucose is available, acetate is not converted to pentose. Using ethanol as substrate in replacement cultures leads to a marked acceleration of acetylene production; under these conditions, acetate-1-C¹⁴ is incorporated both into the C₁₁ acid and into the xylose portion. The percentage of incorporation of acetate-1-C¹⁴ into the acetylenes is virtually equal to the percentage of yield from alcohol consumed, indicating that all of the ethanol is utilized via acetate, or a derivative thereof (138). In a detailed comparison of utilization of acetate-1-C¹⁴ in the presence of 4 per cent glucose or 1.5 per cent ethanol, it was concluded that the increased polyacetylene synthesis corresponds to a proportionately increased turnover of precursor acetate (139).

In *Polyporus anthracophilus*, evidence for the predicted utilization of acetate-1-C¹⁴ for formation of matricaria ester XLIII was obtained by Bu'Lock *et al.* (140); it was also shown that the ester was synthesized *de novo* even in old cultures of up to 63 days in which the ester was simultaneously being destroyed. In some strains of this organism, no longer producing polyacetylenes, large amounts of terpenes, principally eburicoic acid, were formed.



POLYPROPIONATE COMPOUNDS

The macrolide aglycones of some antibiotics may be derived formally entirely from C₃ units (e.g., erythromycin) or from C₃+C₂ units (e.g., methymycin). The utilization of labeled propionate for the biosynthesis of the erythronolide moiety, XLIV, R = R' = H, of erythromycin, XLIV, R = XLVII, R' = XLVI, has been well documented in *S. erythreus* (141 to 144). Chemical degradations indicate that there is no randomization of the propionate carbon atoms, which are used as follows: propionate-1-C¹⁴ → label in 1, 3, 5, . . . 13; propionate-2-C¹⁴ → label in 2, 4, 6, . . . 14; propionate-3-C¹⁴ → label in angular methyls and 15 (see Fig. 9). Using propionate-1-C¹⁴-3-T, Grisebach *et al.* showed in one experiment that 92 per cent of the C¹⁴ was in carbon atoms 1, 3, 5 . . . and 96 per cent of the T was in the angular methyl groups. In other experiments a greater randomization of C¹⁴ was observed (143).

Such experiments clearly establish the role of propionate in the biosynthesis of the erythronolide portion; further evidence against a postulated C₁

addition (60) to acetate units is the fact that formate and methionine do not label erythronolide (142, 143).

The possibility that methylmalonyl CoA is the "active" form of propionate was considered likely (142, 143), and Kaneda & Corcoran (145) have observed that methylmalonate ($C^{14}H_3$) was incorporated into erythromycin with the same isotope distribution as that observed from propionate-3- C^{14} . By analogy with fatty acid synthesis it was to be expected that carbons 15, 14, and 13 would be derived from a "starter" propionyl CoA unit with the rest of the carbon chain from methylmalonyl CoA. Although this group (carbons 15, 14, and 13) contained one-seventh of the total activity in the experiment of Corcoran *et al.* (142), Vaněk and his colleagues find a much higher level of activity in these carbon atoms—carbon 13 containing about

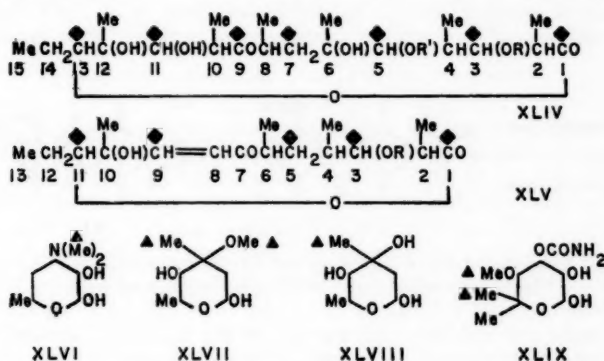


FIG. 9. Macrolide aglycones and sugar components of some antibiotics.

twice as much activity as $C_{1,3,5}$... etc. (144). Vaněk concludes that erythronolide is therefore formed from one molecule of propionyl CoA and six molecules of methylmalonyl CoA.

Grisebach, Hofheinz & Achenbach (146) and Vaněk, Půža, Majer & Doležilová (147) have studied the incorporation of labeled acetates into erythromycin. Although Grisebach *et al.* report a substantial incorporation of C^{14} from acetate (and also from propionate-1- C^{14}) into the nonlactone moieties of erythromycin, Vaněk *et al.* find most of the activity in erythronolide. From acetate-1- C^{14} , 90 per cent of the total activity of erythronolide was in $C_{1,3,5}$... (146, 147). From acetate-2- C^{14} , 90 per cent of the activity was found equally distributed in $C_{2,4,6}$... with the attached methyl groups (including C_{10}) (147). It is apparent that acetate passes through a di- or tricarboxylic acid cycle, and may be converted to methylmalonate via succinate (146, 147).

Tracer studies of methylmycin (XLV, R = XLVI) biosynthesis in *S. venezuelae* show a good incorporation of propionate-1- C^{14} (2.5 per cent of added

isotope) into the aglycone, although acetate-1- C^{14} was poorly incorporated, with extensive randomization. Partial chemical degradations led Birch *et al.* (148) to conclude that only five propionate units were utilized and that carbons 7 and 8 were derived from acetate as shown. The degradations so far presented do not exclude the possibility that carbon 7 is derived from propionate-1- C^{14} , that the activity of carbons 1, 3, 5, 7, and 9 is approximately equal, and that carbon 11 is about 40 per cent higher than the other labeled atoms. This would be analogous to the finding of Vaněk in erythronolide. In this event, all of the carbon atoms of the methylmycin aglycone would be propionate-derived, providing a rationalization for the failure to incorporate acetate. The methyl group originally attached to carbon 8 must be presumed to have undergone oxidation and decarboxylation at some stage. For the aglycone of magnamycin (*S. halstedii*), Grisebach *et al.* have reported briefly that one of the branched methyl groups is derived from propionate (149).

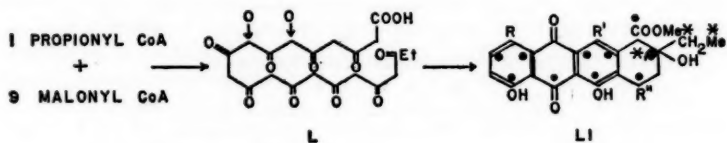
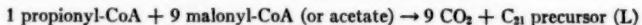


FIG. 10. Biosynthesis of pyrromycinone and related compounds. In this figure, * indicates a minor degree of labeling from acetate-1- C^{14} .

In connection with the sugar moieties of the macrolide and other antibiotics shown in Figure 9, examples of C-methylation from methionine ($C^{14}H_3$), as well as N- and O-methylation, have been demonstrated for desosamine, XLVI, cladinose, XLVII, mycarose, XLVIII, and noviose, XLIX (129, 148, 149, 150).

POLYACETATE + PROPIONATE COMPOUNDS

A study of the biosynthesis of ϵ -pyrromycinone has revealed a combined propionate-acetate (or more likely a propionate-malonate) pathway. ϵ -Pyrromycinone is the aglycone of various antibiotics (e.g., pyrromycin, the cinerubins, the rutilantins) and is identical with rutilantinone (151). Addition of acetate-1- C^{14} to a rutilantin-producing *Actinomycete* culture gave a 0.6 per cent incorporation into ϵ -pyrromycinone, LI, $R = R'' = OH$; $R' = H$; a higher incorporation (1.6 per cent) was obtained with propionate-1- C^{14} . Chemical degradation revealed the pattern shown in Figure 10 (152). From propionate-1- C^{14} , only one carbon was labeled; from acetate-1- C^{14} , nine carbons were equally labeled, and the three carbons designated * also contained small amounts of radioactivity, with random distribution. The biosynthesis of ϵ -pyrromycinone may therefore be represented:



The origin of the methyl group of the ester group is undetermined. It is of interest that the following six compounds, LI, of the propionate-acetate type are apparently known, showing a range of oxidative and reductive steps (153).

7-deoxyaklavinone	LI, R=H	R'=H	R''=H
aklavinone	H	H	OH
ζ-pyrromycinone	OH	H	H
ε-pyrromycinone	OH	H	OH
ζ-isorhodomycinone	OH	OH	H
ε-isorhodomycinone	OH	OH	OH

TETRONIC ACIDS

The biosynthesis of penicillic acid, LIV, has been extensively studied. Birch *et al.* reported that *P. cyclopium* utilized acetate-1- C^{14} (2.3 per cent incorporation) but not mevalonate-2- C^{14} (154). They suggested the possibility that penicillic acid was formed from a derivative of orsellinic acid by oxidation and ring cleavage, similar to that observed with patulin. Mosbach made the decisive observations that orsellinic and penicillic acid were simultaneously formed in *P. baarnense* and that orsellinic acid, labeled at position 2 and in the COOH group, was converted to penicillic acid with C^{14} at position 3 only (47). Hence of the two possibilities, a and b, for orsellinic acid cleavage shown in Figure 11, route b is used, contrary to the suggestion of Birch *et al.* (154). Small amounts of orsellinic acid are also present in *P. cyclopium* cultures producing penicillic acid (20). In a detailed study, Bentley & Keil demonstrated the conversion of glucose to acetate and verified the

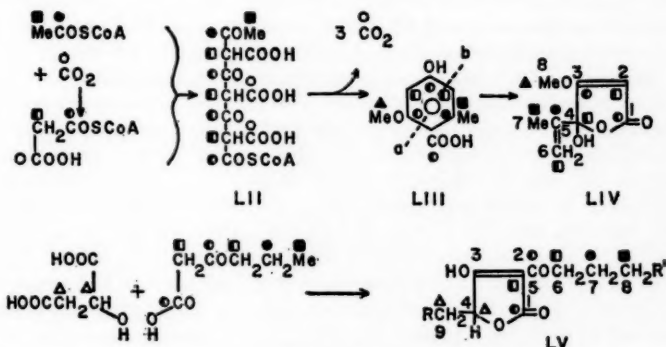


FIG. 11. Pathways postulated for the biosynthesis of tetronic acids.
 Δ = methylene carbon of succinate.

alternate labeling pattern expected for the methyl and carboxyl carbons of acetate (155). C_1 units provide the MeO group, as also do carbon 1 of glucose and particularly carbon 1 of ribose. Furthermore, malonate was well utilized for penicillic acid biosynthesis in *P. cyclopium* (35 per cent incorporation of malonate-2- C^{14} and 9.5 per cent of malonate-1,3- C^{14}). From malonate-2- C^{14} , carbons 2, 4, and 6 each contained 33 per cent of total activity; from malonate-1,3- C^{14} , carbons 1 and 3 each contained about 50 per cent of total activity (20, 155). The labeling pattern was thus strikingly different from that with acetate; carbons 7 and 5 were derived from acetate but not from malonate. Added malonate was therefore not significantly converted to acetate and apparently represented a precursor beyond the acetate level. Condensation of one molecule of acetyl CoA and three of malonyl CoA yields the probably hypothetical intermediate, LII. Loss of 3 CO_2 at some stage, cyclization, and methylation lead to orsellinic acid methyl ether, LIII, and hence to penicillic acid. Mosbach has obtained similar results for orsellinic acid biosynthesis in *P. baarnense* with malonate-2- C^{14} (48).

In a study of the formation of carolic acid, LV, $R=H$, $R'=OH$, and carlosic acid, LV, $R=COOH$, $R'=H$, in *P. charlesii*, Lybing & Reio observed incorporation of activity from acetate-1- C^{14} into carbons 1, 5, and 7 but only a very low incorporation into carbons 3, 4, 9, and 10 (see Fig. 11). They suggested that these structures were derived by condensation of a C_6 moiety obtained from three acetyl units and a C_4 dicarboxylic acid of the citric acid cycle, such as malic acid (156). A major difficulty with this hypothesis is that with acetate as precursor, operation of the citric acid cycle should have given rise to a labeled C_4 dicarboxylic acid. Bentley *et al.* confirmed the lack of incorporation of activity from both acetate-1- and -2- C^{14} into carbons 3, 4, 9, and 10, demonstrated the alternation of carboxyl and methyl labeling in the six-carbon unit, and found that malonate labeled only four of the six acetate-derived carbons (157, 158). Formation of methyl-labeled acetate was demonstrated from glucose-1- and -6- C^{14} . It is apparent that the C_6 precursor is a typical unit formed from one acetyl CoA + two malonyl CoA units.

Succinate-2,3- C^{14} was a good precursor of the tetronic acids, most of the activity being found in carbons 4 and 9. This provides strong evidence for the original suggestion of a C_4 dicarboxylic acid as a precursor, but there is at present no clear-cut explanation for the lack of labeling from acetate. Carlosic acid may be the initial product of the $C_6 + C_4$ condensation, and a likely series is carlosic acid \rightarrow carlic acid, LV, ($R=COOH$, $R'=OH$) \rightarrow carolic acid \rightarrow carolinic acid, LV, ($R=H$, $R'=COOH$). There was poor incorporation of activity from DL-tartrate-1,4- C^{14} into carolic acid. This was of interest (a) in view of Gander's (159) observation that both tartrate and acetate are incorporated into the galactofuranose units of the polygalactofuranose produced by the same organism, and (b) in view of the close structural similarity between the furanoid ring and the tetronic acids. It should be emphasized that the mold tetronic acids, despite the structural resemblance to ascorbic acid, are biosynthesized by a wholly different pathway. There are,

however, many resemblances between the biosynthesis of carolic acid and tenuazonic acid (see page 616).

TERPENE COMPOUNDS BASED ON MEVALONATE

Only a few mevalonate-derived compounds, of special interest, can be considered here. The metabolite, rosenonolactone, LVII, from *Tricothecium roseum* is a diterpenoid compound (160, 161) for which the "biogenetic" isoprene rule (162) requires a methyl migration. Simultaneous studies of the biosynthesis of rosenonolactone by Birch *et al.* (163, 164) and by Britt & Arigoni (165) established the validity of the over-all biogenetic theory, as shown in Figure 12. From mevalonate-2- C^{14} the methyl group (C_{16}) is labeled, but not the carbon of the lactone ring (C_{15}), so that carbons 2 and 3' of mevalonate do not become equivalent at any stage; mevalonate-2- C^{14} also labels carbons 4, 6, and 9. Acetate-1- C^{14} labels carbons 1, 3, 5, 7, 10, 12, 14, and 18. The results agree with the postulated 1,2-methyl shift.

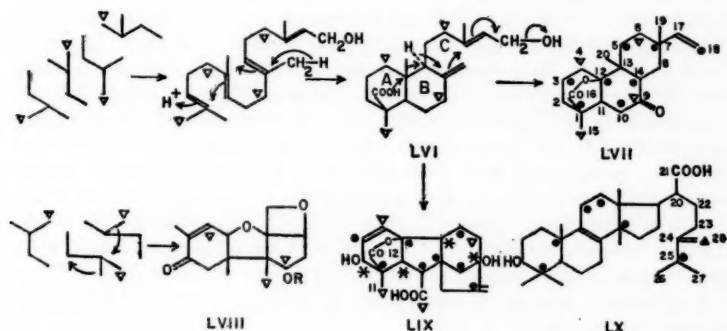


FIG. 12. Compounds derived from mevalonic acid. In this figure, activity from mevalonate-4- C^{14} is indicated by *.

Tricothecin, an antifungal metabolite of *Tricothecium roseum*, has the structure, LVIII, $R = \text{COCH}=\text{CHMe}$; i.e., the isocrotonate ester of the keto-alcohol, tricothecolone, LVIII, $R = \text{H}$ (166). The C_{15} carbon skeleton of tricothecolone can be derived from three isopentane units, if a 1,3- or, alternatively, a double 1,2-methyl group migration takes place. Mevalonate-2- C^{14} is 0.5 per cent incorporated into tricothecin wholly into the tricothecolone moiety (167). The observed C^{14} distribution indicates a double 1,2-methyl migration as shown in Figure 12. Acetate-1- C^{14} was poorly utilized, 95 per cent of the incorporated C^{14} being in the isocrotonate chain.

The chemistry of the gibberellins has recently been reviewed (168), while the biosynthesis of gibberellic acid, LIX, in *Gibberella fujikuroi* has been studied by Birch *et al.* using acetate-1- C^{14} (0.2 per cent incorporation),

mevalonate-2-C¹⁴ (2 per cent incorporation), and mevalonate-4-C¹⁴ (0.13 per cent incorporation) (164, 169, 170). Four molecules of mevalonate or twelve of acetate were implicated; as in rosenonolactone, mevalonate-2-C¹⁴ labels the methyl group, C₁₁, but not the carbon of the lactone ring, C₁₂. The results are in agreement with the formation of the tricyclic diterpene skeleton, LVI, analogous to the rosenonolactone precursor, with the isotope distribution shown in Figure 12. At some stage in the biosynthesis, there is an oxidative loss of the methyl group of the diterpene LVI; i.e. the group which in rosenonolactone becomes C₂₀. Ring B contracts to the structure of a cyclopentane-carboxylic acid, possibly through a 9,10-dioxygenated intermediate. Also, the phyllocladene bridged ring structure is formed from ring C, as originally suggested by Wenkert (171). Biosynthetically labeled gibberellic acid has been used to establish the validity of the Wagner-Meerwein rearrangement for the isomerization of allogibberic acid to gibberic acid (170, 172).

Redemann & Meuli observed an increased production of gibberellin in *Fusarium moniliforme* cultures treated with β -methylcrotonic acid; activity from β -methylcrotonate-3-C¹⁴ was incorporated into gibberellic acid (173). They suggested that β -methylcrotonate was a direct precursor. Similarly, Zweig & DeVay have investigated the utilization of 3-methylcrotonic acid-3-C¹⁴ and methylcrotonic acid (both methyl groups labeled) in *F. moniliforme*, as well as that of labeled acetates, mevalonate-2-C¹⁴, and other compounds (174). It is surprising that in replacement cultures acetate-1 and 2-C¹⁴ are incorporated into gibberellic acid, but not uniformly labeled glucose, fructose, or sucrose, or mevalonate-2-C¹⁴. In experiments in which precursors were added to the original medium, there was again no utilization of added mevalonate, whereas the labeled crotonic acids were utilized even more effectively than labeled acetates. The failure of mevalonate-2-C¹⁴ to label gibberellic acid in *F. moniliforme* is striking in view of the result obtained by Birch *et al.*

The biosynthesis of eburicoic acid, LX, has been studied in *Polyporus sulfureus*. The compound is a tetracyclic triterpene and is of importance in view of its relationship to lanosterol and cholesterol. The mold was grown as a surface culture for three to four months, and the incorporation of acetate-1-C¹⁴ and formate-C¹⁴, added at the time of inoculation, was 2 and 1.7 per cent, respectively (175, 176). Partial degradations established that activity was incorporated as shown in Figure 12 in agreement with the utilization of acetate as a two-carbon unit, through the squalene pathway. The methylene carbon, C₂₈, was not derived from acetate-1-C¹⁴; with labeled formate, however, 60 per cent of the total activity was in C₂₈, while the remainder was distributed uniformly over the other carbon atoms.

COMPOUNDS DERIVED DIRECTLY FROM HEXOSES

Both reductions of sugars to sugar alcohols and oxidations to aldonic or ketoaldonic acids are well known in molds, and will not be discussed here. The elegant crystallization of the glucose oxidase from *P. amagasakiense* by

Kusai and his colleagues (177, 178) is, however, particularly noteworthy. Similarly, of considerable interest is the report that a purified galactose oxidase from *Polyporus circinatus* oxidizes D-galactose at C₆, rather than at C₁, to form D-galacto-hexo-1,6-dialdose (179). Some other mold products, derived directly from glucose without cleavage of the carbon chain, are relevant to this review.

Since kojic acid, LXI, can be formed in good yields by growth of various molds on substrates with three carbon atoms, it has often been argued that hexoses were first cleaved to triose in this biosynthesis. The experiments of Arnstein & Bentley (180, 181) indicated clearly that this was not so, and that glucose was used without cleavage in *A. flavus-oryzae* as shown in Figure 13. A similar conclusion was reached by Denison, Carson & Foster with *A. flavus* (182). Little is known about the general biochemistry of these organisms. At least two enzymes of the Embden-Meyerhof pathway (aldolase and triose phosphate isomerase) are present in *A. flavus-oryzae*, although from glucose-1-C¹⁴ the initially higher activity of respiratory CO₂ in the early stages of

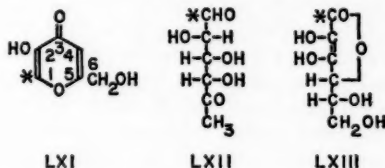
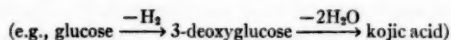


FIG. 13. Compounds derived from hexoses without cleavage of the carbon chain. * = label from glucose-1-C¹⁴.

growth suggests the hexose monophosphate pathway (181, 183). Further, there is little evidence for the role of possible phosphorylated intermediates in the conversion of glucose to kojic acid. Arnstein & Bentley concluded that low levels of phosphate in the medium affected both the formation and reutilization of kojic acid by an indirect effect on general metabolic processes (183). The utilization of phosphate compounds of the mycelium during synthesis and break-down of kojic acid in *A. tamarii* and *A. oryzae* has been studied recently (184). In a series of experiments with acetate-1- and 2-C¹⁴, glycine-1- and 2-C¹⁴, pyruvate-2- and 3-C¹⁴, acetone-1,3-C¹⁴, dihydroxyacetone-2-C¹⁴, and L-lysine- α -C¹⁴, it was found that carbons 4, 5, and 6 invariably contained three times as much activity as carbons 1, 2, and 3 (16, 181). A "reversal" of this pattern was found using D-ribose-1-C¹⁴ as the substrate for growth: in this case the percentages of total activities in kojic acid were: C₁, 36.7; C₂, 18.9; C₃, 24.5; C₄₋₆, 20.0 (185). These isotope distributions from 1-C¹⁴-ribose indicated clearly that ribose was metabolized to a C₆ precursor, by the transketolase-transaldolase pathway, and that the C₆ units were then converted to kojic acid. The fact that these reactions were available also explains the observations with dihydroxyacetone-2-C¹⁴. This

compound is presumed to react with unlabeled sedoheptulose phosphate by the transaldolase reaction to yield a C_6 unit labeled principally in carbon 5. Similarly, with the other "small molecules," conversion to a C_6 unit followed by the transaldolase reaction yields kojic acid mainly labeled in carbons 4, 5, and 6.

Despite the detailed tracer experiments, the precise mechanism for kojic acid synthesis is still unknown. The simplest route involves oxidation and dehydration



but the frequent accumulation of gluconic and ketogluconic acids in fungi has suggested the possible involvement of such acids in kojic acid biosynthesis.

The biosynthesis of 5-keto-6-deoxyarabohexose, LXII, an unusual sugar present in Hygromycin A formed by *S. hygroscopicus*, has been studied (186). The sugar could not be isolated as such, but as L-fucose diethylmercaptol after reduction of the antibiotic with sodium borohydride. Almost all of the activity was in C_1 of the keto sugar from glucose-1- C^{14} , in C_2 from glucose-2- C^{14} , and in C_6 from glucose-6- C^{14} . These results are consistent with the direct conversion of the carbon chain of glucose, as shown in Figure 13.

Although it has not been experimentally demonstrated, the other sugars from antibiotics, discussed earlier, presumably have a C_6 chain derived from glucose, and with the C_1 additions previously noted in Figure 9. The branched-chain pentose, cordycepose, found in cordycepin (*Cordyceps militaris*) is obviously not directly derived from glucose, but a relationship to the hexose monophosphate pathway has been suggested. Acetate, isovalerate, and ribose are not precursors for cordycepose (187).

Many species of *Penicillia* have been reported to form D-araboascorbic acid, LXIII, from D-glucose (188). Labeled araboascorbic acid was isolated and degraded after growth of *P. notatum* on glucose-1- C^{14} . Seventy-eight per cent of the total activity of the araboascorbic acid was in C_1 (see Fig. 13). It was concluded that there is a direct conversion of glucose, without fragmentation and without the "inversion" of the carbon chain that is observed in the animal.

A unique conversion is that of D-glucose to N-methyl-L-glucosamine by *S. griseus* in which an enantiomorphic change of the entire molecule must occur. Silverman & Rieder have shown that from glucose-1- C^{14} , 78 per cent of activity in the amino sugar is in C_1 ; similarly, from glucose-6- C^{14} , 92 per cent of amino-sugar activity is in C_6 (189). It is concluded that the carbon chain of glucose is used without inversion and without cleavage, although the precise mechanism is unknown.

COMPOUNDS RELATED TO THE TRICARBOXYLIC ACID CYCLE

Citric acid.—Although citric acid formation in molds has been extensively investigated, the changes in specific activity of the tricarboxylic acid cycle

enzymes with the fermentation time are of unique interest from the point of view of "shunt" metabolism (8). With cell-free preparations from shaken cultures, all of the tricarboxylic acid cycle enzymes were found during the first 24 hours, with no citrate present. After 46 hours, with citrate accumulating, the specific activity of the condensing enzyme increased, eventually to about 10 times the 24-hour level, while isocitric dehydrogenase could not be detected beyond the 24-hour period and aconitase was not present after 46 hours. Evidence that citrate inhibits isocitric dehydrogenase was also obtained. These observations clearly indicate that citrate accumulation is a consequence of the interruption of the cycle at the aconitase and isocitric dehydrogenase levels.

Itaconic acid.—It was long suspected that itaconic acid, LXIV, was a decarboxylation product of cis-aconitic acid. However, results from detailed tracer studies indicated that the proposed reaction, $>C=CHCOOH \rightarrow >C=CH_2$, did not take place. In a short-term experiment of 24 hours, 60 per cent of activity from acetate-2- C^{14} was in C_5 of itaconic acid, rather than in C_3 , as expected on the basis of the known reaction mechanism for aconitase (15, 190). Furthermore, methylene- and carboxyl-labeled succinates were incorporated into itaconic acid without decarboxylation (see Fig. 14) (191).

This difficulty was resolved by a study of the reaction mechanism of the aconitate decarboxylase, first obtained in cell-free extracts by Bentley & Thiessen in 1955 (9, 192) and by Jenssen, Larsen & Ormerod in 1956 (193) from *A. terreus*. The preparations also contained aconitase and decarboxylated cis-aconitic acid, (+)-isocitric acid, and citric acid in that order of effectiveness (9, 192, 193, 194). Tracer studies demonstrated that the reaction mechanism of the aconitase component was the same as that of mammalian aconitase, but that the aconitate decarboxylase was actually a β, γ -, rather than an α, β -decarboxylase (9, 195). Furthermore, decarboxylation of cis-aconitic acid in D_2O gave itaconic acid with one atom of deuterium at position 3, suggesting the mechanism shown in Figure 14 (9).

From a detailed study of the influence of medium pH on itaconic acid formation, Larsen & Eimhjellen (196) concluded that an acid environment is necessary for the synthesis of an essential enzyme system, presumably the aconitate decarboxylase. The aconitate decarboxylase has been partially purified by ammonium sulfate precipitations, but the authors do not indicate whether the preparation still contains aconitase (197, 198). Studies of the early stages of glucose dissimilation indicated a major role for the Embden-Meyerhof pathway; pyruvate accumulates in the presence of arsenite; fluoride and iodoacetate inhibit glucose utilization; aldolase is present; and the respiratory CO_2 from glucose-1- C^{14} has a low C^{14} content initially, later increasing to a constant value (191, 199, 200). The difficulties of defining a precursor role for citrate and aconitate in intact cells, by means other than tracer techniques, have been recognized and discussed (199, 200). From experiments in intact cultures, Bhargava *et al.* have, however, concluded that

the major part of the itaconic acid is formed directly from pyruvate without the prior formation of acetate or citrate, or both (201). In these experiments, it was observed that (a) acetate at 2 per cent concentration inhibits itaconic acid formation, although not the utilization of cane sugar; and (b) pyruvic acid is a better substrate than cane sugar. These experiments, while hard to evaluate at present, are certainly not incompatible with the pathway, pyruvate \rightarrow acetate \rightarrow citrate \rightarrow aconitate \rightarrow itaconate. In any case, the direct utilization of pyruvate by condensation of two molecules, followed by dehydration and oxidative decarboxylation (202), is not consistent with the tracer studies.

Itatartaric acid.—Mutants of *A. terreus* obtained after ultraviolet irradiation form itatartaric acid, LXV, as well as itaconic acid (203, 204, 205). From such mutants, Arpai (205) has isolated cell-free extracts of the enzyme,

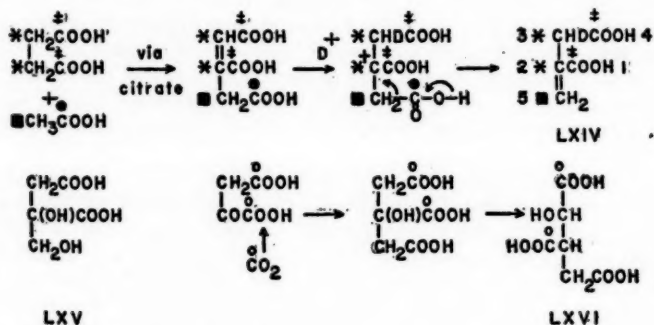


FIG. 14. Compounds related to the tricarboxylic acids. In this figure, the carboxyl carbon of succinic acid is indicated by ‡ and the methylene carbon of succinic acid by *.

itaconic oxidase, which in the presence of oxygen converts itaconic acid to itatartaric acid. The enzyme has a pH optimum of 4.0 to 4.2 and is unaffected by dialysis or by the presence of CN^- , NH_2OH , or iodoacetic acid. Itatartaric acid and its lactone have also been identified in *A. itaconicus*, and it was suggested that these compounds were derived directly from citrate rather than via itaconic acid (206).

Alloisocitric acid.—An interesting development has been the identification of L8-alloisocitric acid, LXVI, as a major product from various *Penicillia* when a neutral growth medium is used (207). The production of the alloisocitrate is not inhibited by the aconitase inhibitor, fluoroacetate, in *P. purpurogenum* var. *rubrisclerotium*. In a tracer study, Beppu, Arima & Sakaguchi (208) observed that C^{14}O_2 is incorporated equally and exclusively into C_1 and C_6 ; alloisocitrate, citrate, and malate isolated in the presence of fluoroacetate had the same specific activity, 50 per cent of this being in C_6 .

with both allosictrate and citrate. Citrate-1,5- C^{14} was converted to allosictrate (12 per cent yield); the labeling in the final products was as follows, the first figure referring to percentage of total activity with allosictrate, the second to citrate: C_1 , 64.3, 60.1; C_6 , 11.8, 13.5; $C_{2,3,4,5}$ 29.4, 30.5. It was suggested that allosictritic acid is formed from citrate, and that some recycling takes place since activity is located in C_6 and there is dilution in C_1 . The presence of condensing enzyme, aconitase, and fumarase was demonstrated. The exact nature of the conversion of citrate to allosictrate is unknown. An interesting possibility is that the biosynthesis of allosictrate may involve the formation of cis-aconitate from citrate, perhaps by an enzyme of the "aconitic hydrazase" type. In *A. niger*, Nielson has provided clear evidence for the presence of an aconitase of the usual type, as well as "aconitic hydrazase" forming only citrate from cis-aconitic acid (209). If aconitic hydrazase is not inhibited by fluoroacetate, the action of this enzyme and a subsequent rehydration of aconitate would account for the biosynthesis of allosictrate.

Tartaric and related acids.—Although tartrate is a component of the familiar Raubin-Thom medium and is stimulatory to *N. crassa* (210), little is known about its metabolism in molds. As noted in connection with tetroneic acid synthesis, DL-tartrate-1,4- C^{14} is converted to the polysaccharide, galactocarolose (159). Although *P. cyclopium* produces little or no penicillic acid unless tartrate is present, C^{14} from labeled tartrate is not incorporated into this metabolite (155). In *A. niger*, tartrate is quantitatively converted to oxalic acid (211). A strain of *A. fumigatus* produces large amounts of the related trans-L-epoxysuccinic acid (212), while 1-ethoxy-1,2-ethylenedicarboxamide is accumulated by a *Streptomyces* strain (213).

INCORPORATIONS OF AMINO ACIDS

Peptides, or peptide-containing materials, are often found in fungi. Wieland has discussed possible biogenetic pathways for the oligopeptides comprising the toxins of *Amanita* species (214) while Katz has summarized the biosynthesis of the actinomycin peptides (127). The following amino acids are used directly in formation of nonpeptide-shunt metabolites.

Simple amino acids.—Several cyclic hydroxamic acids, or related compounds, are theoretically derivable from two amino acids. MacDonald has shown that *A. flavus* incorporated DL-leucine-1- C^{14} directly into aspergillic acid, LXVII, and that the second amino acid, isoleucine, is not formed from leucine (see Fig. 15). With a preparation of uniformly labeled isoleucine (containing 15 per cent of leucine), most of the activity was located in the isoleucine and allosileucine obtained on degradation, and only a trace in leucine (215).

Valinomycin, formed in *S. fulvissimus*, contains two L-lactyl-L-valyl and two D- α -hydroxyisovaleryl-D-valyl radicals linked in a ring by alternating amide and ester bonds. MacDonald has found that L-valine-1- C^{14} (but not the D enantiomorph) was incorporated equally into both D and L-valyl

moieties, to a lesser extent into the D- α -hydroxyisovaleryl portion, but not at all into the lactyl portion. Carbon 14 was present only in the COOH groups (216).

Stickings & Townsend have observed the direct utilization of L-isoleucine (21 per cent incorporation) as a precursor for tenuazonic acid, LXVIII, a metabolite of *Alternaria tenuis* (217). Carbons 2, 3, 6, and 7 are derived directly from acetate, and in this respect the compound resembles carolic acid. A minor and equal labeling (about 2 per cent of the total) in C₄ and C₁₀ from acetate-1-C¹⁴ is consistent with the formation of isoleucine via the oxaloacetate, α -ketobutyrate pathway described in *Torulopsis utilis* (218).

A possible role for glycine in the biosynthesis of phomazarin, LXIX, a compound formed under ill-defined conditions in *Phoma terrestris*, has been

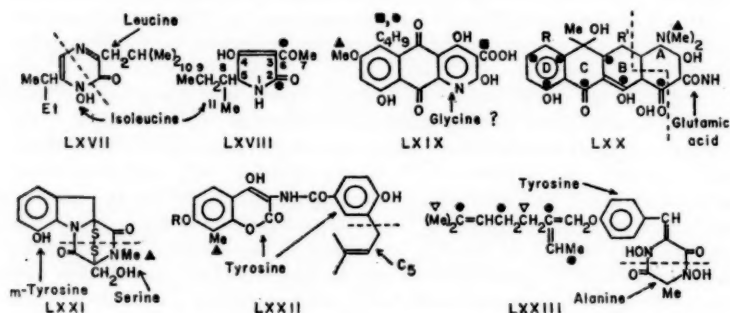


FIG. 15. The utilization of various amino acids for biosynthesis of shunt metabolites.

suggested⁴ (89). The incorporation of probably eight acetate units was observed, as well as the utilization of formate for the OMe group.

Glutamic acid.—A predominant role for small molecules in the biosynthesis of chlorotetracycline, LXX, R = Cl, R' = H, in *S. aureofaciens* was demonstrated in 1956 (219). The acetate molecule was used intact, while glycine-2-C¹⁴ and methionine-C¹⁴H₃ were used remarkably well (52 and 48 per cent, respectively). From the glycine-2-C¹⁴ experiment, 40 per cent of total activity was found in the 4'-dimethylamino group. The meager information relating to the chlorination step in chlorotetracycline biosynthesis has been reviewed (220); it has also been observed that the chlorine of chlorofatty acids can be utilized by *S. aureofaciens* (221).

Similarly, Snell *et al.* demonstrated the incorporation (up to 6 per cent) of acetate-2-C¹⁴ into oxytetracycline, LXX, R = H, R' = OH (*S. rimosus*), less C¹⁴ being found in ring A than in B, C, or D (222). Snell *et al.* later observed

⁴ The diagram accompanying the original article indicates that the following unit, containing three carbon atoms, is derived from glycine; C-C-N-C. The origin of the third carbon atom is not discussed.

the incorporation of methionine- $C^{14}H_3$ into oxytetracycline, with activity only in the 6-methyl group and the $N(Me)_2$ group. Partial degradations of oxytetracycline from acetate-2- C^{14} indicated a major role for this precursor in rings B, C, and D; and from glutamate-2- C^{14} , 95 per cent of total activity was incorporated into ring A (223). The suggested mechanism is shown in Figure 15.⁵

An interesting cofactor, cosynthetic factor I, has been shown to be involved in the production of chlorotetracycline by *S. aureofaciens* S-1308. In the absence of this factor which is elaborated by many strains of *S. aureofaciens* and which has been obtained crystalline from mutant W-5, the mutant S-1308 normally accumulates 7-chloro-5a(11a)-dehydrotetracycline (224, 225). The cofactor, $C_{19}H_{22}N_4O_8$, is a stable, water-soluble, yellow material, readily reduced by aqueous sodium borohydride to a colorless product, reoxidized by air. The absorption spectrum (maxima at approximately 245, 290, and 420 m μ in 0.1 N NaOH) suggests that the factor may be related to the pteridines or flavins. It is considered to be a cofactor for one (or more) hydrogen transfer systems.

Aspartic acid.—From DL-aspartic acid-4- C^{14} , Birch *et al.* obtained a 2 per cent incorporation of activity into β -nitropropionic acid, using *P. atrovenetum*, located entirely (96 per cent) in the carboxyl groups of the metabolite. On the other hand, β - C^{14} -alanine was not converted to β -nitropropionic acid (226). Hylin & Matsumoto have reported that in the presence of NH_4^+ ion, C_4 dicarboxylic acids added to Raulin-Thom medium at a concentration of 2 mmoles per 100 ml of medium stimulate the formation of β -nitropropionic acid (227). Aspartic acid is less efficient than fumaric or succinic acids. Furthermore, in the absence of additional NH_4^+ ion, aspartic acid does not increase the yield. It was expected that if aspartate were a direct precursor of β -nitropropionic acid, the αNH_2 would be oxidized *in situ* without a further requirement for NH_4^+ ion. Further tracer experiments with aspartic acid labeled with both N^{15} and C^{14} might help to reconcile these conflicting observations.

Aromatic amino acids.—The structure for gliotoxin, LXXI, was revised in 1958 (228), and the pathway for gliotoxin biosynthesis in *Trichoderma viride* is believed to be as follows on the basis of tracer experiments with various C^{14} , and T-labeled phenylalanines, DL-metatyrosine-T, DL-serine-1- and 3- C^{14} , glycine-2- C^{14} and DL-methionine- $C^{14}H_3$; phenylalanine \rightarrow metatyrosine \rightarrow dethiogliotoxin \rightarrow gliotoxin. Both serine and methionine are involved in the formation of the gliotoxin structure from metatyrosine. Hydroxylation of phenylalanine apparently precedes cyclization since DL-tryptophan-7a- C^{14} , although incorporated into the mycelium, does not give rise to labeled gliotoxin (229, 230).

⁵ Gatenbeck has concluded that glutamate is not preferentially utilized for the formation of the carboxamide group or any part of ring A of oxytetracycline. Instead, a carboxylation reaction is apparently responsible for the carboxamide group. Malonate probably constitutes the condensing principle for formation of the whole carbon skeleton. See *Biochem. Biophys. Research Commun.*, **6**, 422 (1962).

The biosynthesis of a coumarin ring found in the aglycone of novobiocin, LXXII, R = XLIX (*S. niveus*), involves the intact utilization of L-tyrosine (231). The additional C-Me group is derived by a methyl transfer from L-methionine (129). Further, L-tyrosine provides seven of the carbon atoms in the benzenoid moiety. Mycelianamide, LXXIII, is also derived from tyrosine itself and alanine and has certain structural features in common with gliotoxin. In addition a side chain derived from acetate or mevalonate is present. With acetate-1-C¹⁴ all of the activity was present in the side chain (61, 62).

A role for tryptophan in the formation of ergot alkaloids related to lysergic acid, LXXIV, has been well established (232 to 238); it is known that the carboxyl group is lost in the biosynthesis (233), that tryptamine is not formed prior to alkaloid synthesis (238), and that DL-5-hydroxy-tryptophan (β -C¹⁴) is not a precursor (236). The N-methyl group is derived from methionine-C¹⁴H₃ (or formate) (238). The derivation of five carbons from mevalonate

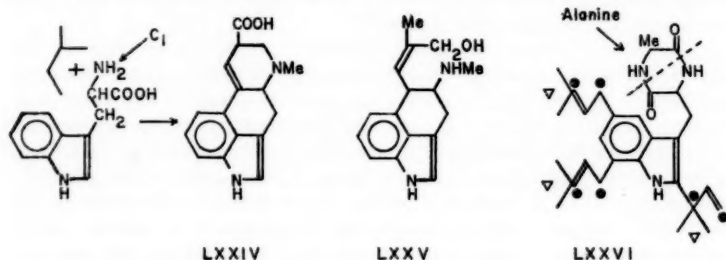


FIG. 16. Compounds derived from tryptophan and mevalonic acid.

nate was shown by Gröger *et al.* (for elymoclavine) (239), by Taylor & Ramstad for lysergic acid (in ergotamine, ergosine, and ergometrine) (240), and by Birch *et al.* for agroclavine and elymoclavine (241). The general outlines for structures of this type are therefore as shown in Figure 16. The interesting compound chanoclavine, LXXV (242), apparently may represent an intermediate stage in the pathway to alkaloids of the lysergic series.

Echinulin, LXXVI (from *A. echinulatus* or *A. amstelodami*), is apparently derived from tryptophan. The structure for echinulin was recently deduced by Quilico and colleagues (243) while the presence of three isoprene units was suggested by the biosynthetic experiments of Birch *et al.* with mevalonate-2-C¹⁴ (6). All of the activity was in the gem-dimethyl groups. Similarly for acetate-1-C¹⁴, the predicted labeling was observed in the isoprene units. Echinulin provides another example (see also atrovnetin and related compounds) for the "reverse" addition of an isoprene unit. From DL-alanine-1-C¹⁴, 65.8 per cent of total activity was in the diketopiperazine ring, confirming the previous suggestion that this amino acid was a precursor of echinulin (244).

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IMMUNOCHEMISTRY¹

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The topics to be discussed in this chapter cover only a small part of the rapidly growing subject of immunochemistry. Fortunately, a number of reviews (71, 114, 116, 146, 159), proceedings of symposia (2, 36, 117, 145, 212, 247), and books (125, 195) have been published recently which help to fill in many of the deficiencies of this article.

ANTIBODIES AND γ -GLOBULIN

Purification:—The purification and fractionation of γ -globulin and antibodies is an unusual problem in that whatever criteria (biological, chemical, or physical) are applied the products are still heterogeneous and it is therefore important to define what is attempted and what is achieved. Purification of an antibody is usually taken to mean an attempt to isolate material all of which will react specifically with the antigen. This reaction may be detected by the formation of a precipitate or other evidence of antibody-antigen interaction. A general method for the isolation of antiprotein antibodies has been proposed by Singer, Fothergill, & Shainoff (217). These authors introduced several sulphhydryl groups into protein antigens by reaction with acetylhomocysteine thiolactone and found that with the proteins bovine serum albumin, ovalbumin, and ribonuclease there was little loss of antigenic activity. The specific precipitate formed was dissociated at pH 2.4 and, on addition of a bifunctional organic mercurial, the thiolated antigen cross linked and precipitated, leaving antibody in solution. This was 98 per cent precipitable and recoveries varied from 25 per cent to 75 per cent depending on the antigen used. A number of methods applicable to particular antibodies have been described, such as that of Farah, Kern, & Eisen (78) for the isolation of rabbit antidinitrophenyl antibody. In this method, dissociation of the antibody and dinitrophenyl bovine γ -globulin is brought about by incubation at neutral pH with hapten, which competes for the combining sites. Antigen is precipitated by addition of streptomycin, and the antibody-hapten complex separated by ion exchange chromatography and salt precipitation. There was a 40 per cent recovery of material which was 90 per cent precipitable by the antigen. Another purification of anti-hapten antibody (anti- β -D-galactosyl-phenylazo serum albumin) was achieved by dissociation of the specific precipitate with hapten followed by precipitation of the antigen at pH 4.7 and separation of the hapten from antibody on Sephadex G.25

¹ The survey of literature for this review was completed in September 1961.

at pH 6.8 [Bassett, Beiser, & Tanenbaum (18)]. An isolation of an antienzyme antibody (anticarbamylphosphate synthetase) depended on the denaturation of the enzyme under the conditions in which the specific precipitate was dissociated (pH 3.2) [Marshall & Cohen (152)]. The most simple method so far used is probably that of Arnon & Sela (6) who isolated rabbit antigelatin antibody by treatment of the specific precipitate at neutral pH with collagenase. This very specific enzyme hydrolyzed the gelatin and destroyed its affinity for antibody which was recovered with full activity. If the antigen was polytyrosyl gelatin, the hapten remained bound to the antibody and could not be separated.

The use of a specific adsorbant for the purification of antibodies has obvious attractions, and many attempts have been made to prepare such material by attaching antigens, usually proteins, to some inert and insoluble compounds. [For earlier work see Isliker (122).] There are three major problems to be overcome: (a) the antigen must be bound irreversibly to the insoluble support, in sufficient quantity and without changing its specific affinity for the antibody; (b) the adsorbent must have a high capacity for antibody without any nonspecific affinity for other serum proteins; (c) the conditions of elution must be such that all the adsorbed antibody is released without denaturation, preferably leaving the specific adsorbent available for re-use. Several recent attempts to use antigen coupled to diazoamino polystyrene have been described [Manecke & Gillert (150); Gyenes, Rose & Schon (106)]. The principal difficulties are that the resin readily adsorbs protein, some of the supposedly coupled antigen is not covalently bound and hence leaches off during elution of the antibody, and also nonspecific adsorption of serum proteins occurs. Yagi *et al.* (248) found that, although serum albumin may be adsorbed rather than covalently bound, elution conditions for the antibody could be found where the antigen remained fixed. They reduced the nonspecific adsorption of serum protein by preincubation of the resin with nonimmune serum. For preparative purposes these conditions were not very satisfactory, as on elution at pH 3.4 only about one-third of the antibody was recovered. Gyenes & Schon (107) found similar recoveries, again using serum albumin as antigen, and also observed that the higher the number of amino groups on the polystyrene resin, the less nonspecific adsorption occurred. Webb & Lapresle (245) found that with such a resin some rabbit antihuman serum albumin was not adsorbed at neutral pH, some eluted at pH 3.5, and more eluted in N/10 HCl. Immuno-electrophoretic studies with partial hydrolysates of serum albumin suggested that the three antibody fractions differed in their specificity.

Gourvitch, Kapner & Nezlin (95) have modified Campbell's method (41) of attaching protein antigens to cellulose. The Russian workers used cellulose powder prepared according to Flodin & Kupke (81), and reacted this with *m*-nitrobenzyloxymethyl pyridinium chloride. The nitro group was reduced, diazotized, and reacted with the protein antigen. Unreacted diazo groups

were blocked with β -naphthol. This preparation proved to be a highly specific adsorbent and was used to isolate and fractionate rabbit antibodies and to study their properties. The capacity of the adsorbent was of the same order as that of previously described adsorbents (about 4 mg antibody/g adsorbent) and the chief interest in the method lies in that a very recent modification has raised the capacity by one hundredfold without loss of specificity [Nezlin *et al.* (164)]. A full account has been published [Gourvitch *et al.* (97)], but is not yet available to the reviewers.

The alternative problem of separating γ -globulin containing antibody into several fractions has been studied either by zone electrophoresis or by chromatography on ion exchange cellulose. The results appear to show a difference between the behaviour of immune γ -globulin from rabbit and human serum. Using carboxymethyl cellulose, Sober & Peterson (221) were able to get a partial separation of human γ -globulin into a number of fractions in which several antibacterial antibodies were unevenly distributed. Similar results were obtained in a fractionation on diethylaminoethyl cellulose [Fahey & Horbett (75); Fahey & Morrison (76); Fahey (74); Abelson & Rawson (1)]. In some cases, but not all, the differences in behaviour of the antibodies were related to the association of the antibodies with S_7 or S_{19} globulin fractions.

In the rabbit, a fractionation of antibodies of different size has been obtained. Thus, Stelos & Talliaferro (227) showed that the S_{19} antibodies to sheep erythrocytes are associated with the γ_1 -globulins and can be separated by zone electrophoresis from the S_7 antibodies against the same complex antigen. However, when the γ_1 -globulin from a rabbit strongly immunized with pneumococcus type III was separated into several distinct components by zone electrophoresis or chromatography on ion-exchange cellulose, the antibody content was the same in each fraction [Askonas, Farthing & Humphrey (11)].

Amino acid content.—Several new amino acid analyses of rabbit antibodies are in close agreement with the results for antipolysaccharide antibodies obtained by Smith *et al.* (219). Fleischer *et al.* (80) found no differences between antibodies to bovine γ -globulin coupled either to a basic or an acidic hapten and Askonas *et al.* (11) found very similar values for the anticarbohydrate antibodies present in different electrophoretic fractions. Such differences as exist between the analyses from several laboratories are probably not significant. The only exception is a report that γ -globulins from human and rabbit sera contain appreciable amounts of hydroxylysine, 0.85 per cent and 0.60 per cent of total nitrogen respectively [Waldschmidt-Leitz *et al.* (242)]. This amino acid has been reported as present in trypsin (1 mole/mole protein) [Viswanatha & Irreverre (241)], but otherwise it is believed to occur only in collagenous proteins [see Greenstein & Winitz (100)]. The presence of 10 to 12 moles hydroxylysine/mole γ -globulin might be of considerable importance in determining biological activity but, so far, attempts in this lab-

oratory to confirm these results have been unsuccessful (Crumpton, unpublished).

The occurrence of chemical differences between different antibodies has frequently been discussed in connection with theories of antibody formation. It is now clear that if they exist, they are small, and an unequivocal solution may depend on detailed knowledge of the chemical structure. A possible short cut is through study of the peptide patterns produced by electrophoresis and chromatography of enzymic hydrolysates of different purified antibodies. This technique has been remarkably successful in picking out very small differences of amino acid sequence between normal and pathological haemoglobins [Ingram (120)]. The technical difficulties are great [Sanger (204)] and they increase rapidly with the size of the protein so that success with a molecule of 170,000 molecular weight is uncertain. However, two such attempts have now been reported. In one of these attempts Gitlin & Merler (93) have compared the peptide patterns of rabbit antibodies specific for different types of pneumococcal polysaccharide using as starting material either the specific precipitate or antibody dissociated from it by strong salt solution [Heidelberg & Kendall (112)]. Subtilisin was the principal enzyme used for digestion, and the antibodies were either oxidized with performic acid, heat denatured, or left native before digestion. After electrophoresis and chromatography, 100 to 110 peptide spots could be distinguished. With the performic acid oxidized material, no reproducible difference in the patterns was observed. The digest of heat-denatured antibodies against the different pneumococcal types showed differences in several peptides, as also did the digest of native antibodies. In such experiments, the digestion must go to completion or spurious differences will be found and the authors do not come to a definite conclusion as to whether their results prove or disprove the existence of a single unique amino acid sequence in all rabbit antibodies. The pieces I, II, and III obtained by papain digestion of the pneumococcal antibodies were also examined by the same methods, and in this case differences were found between the pattern from a digest of both pieces I and II from different antibodies. Again the authors were very cautious in the conclusions which they drew from these results.

Gourvitch *et al.* (96) compared the peptide patterns prepared in a similar way from rabbit antihorse serum albumin and γ -globulin from nonimmune serum. The protein was either native or was denatured with 5 per cent trichloroacetic acid before digestion for 2 hours at 37° pH 8 with 1 per cent trypsin followed by a further 2 hours digestion after adding 1 per cent chymotrypsin. From the digest of denatured protein 40 peptides were separated and a difference of one peptide spot between the inert γ -globulin and the antibody was observed. Many more than 40 different peptides would be expected, and there seemed to be no certainty that digestion with each enzyme was complete. Again it is difficult to draw definite conclusions from these results. A further difficulty in this work arises from the discovery of genetically

controlled differences of structure of γ -globulin from individual rabbits—the allotypes [Oudin (176)]. This phenomenon might be responsible for differences of amino acid sequence between γ -globulin preparations that are unrelated to the presence or absence of antibody combining sites.

Carbohydrate content.—The carbohydrate of human γ -globulin has been analyzed [Rosevear & Smith (202)] and found to be made up of the following residues: galactose 3, mannose 5, fucose 2, glucosamine 8, sialic acid 1. This carbohydrate was attached to the C terminal aspartic acid of the peptide $\text{H}_2\text{N}\text{Glu}\cdot\text{Glu}\cdot\text{Asp}\cdot\text{NH}_2\cdot\text{Tyr}\cdot\text{Glu}\cdot\text{Asp}$. Unless this is a repeated sequence there would appear to be only one carbohydrate group present in each molecule of γ -globulin. This finding appears to be in conflict with the observations of Fahey & Horbett (75) that the hexose content of human γ -globulin increases from 1.1 per cent to 2.3 per cent, depending on the electrophoretic mobility of the fraction. The 19S macroglobulins have a higher carbohydrate content (5 per cent) but the variable results with the 7S globulins were not due to contamination with macroglobulins. These results might be explained by the assumption that the γ_1 -globulins contain two such glycopeptides as isolated by Rosevear & Smith (202), the γ_2 -globulins contain only one, and the intermediary values arise from mixtures of these two types. In a similar analysis of the hexose content of rabbit γ -globulin fractions with different electrophoretic mobilities, Askonas *et al.* (11) found no significant variations.

Enzymic digestion.—The structural basis of antibody activity has been investigated by studying biologically active fragments of antibody obtained by partial hydrolysis. This method has been used with some success over many years, and has recently aroused renewed interest [for earlier work see Porter (189)]. It had been observed [Porter (187)] that crystalline papain would cause very limited hydrolysis of rabbit γ -globulin to produce three pieces separable by chromatography. Pieces I and II (molecular weight 50,000) each contained an antibody-combining site while III (molecular weight 80,000) carried most of the antigenic sites of the original molecule. The presence of one combining site in each of pieces I and II was inferred from their ability to combine with, but not precipitate, the antigen. Equilibrium dialysis studies using antihapten antibody [Karush (129); Nisonoff *et al.* (169)] have confirmed this and shown that little if any alteration of the antibody-combining site was caused by the digestion. It was also found that fragments similar to pieces I and II could be produced by peptic hydrolysis, followed by reduction [Nisonoff *et al.* (166, 170)]. Peptic digestion at pH 5 gave a 100,000 molecular weight fragment which would still precipitate with the antigen, but treatment of this product with cysteine gave two 50,000 molecular weight pieces which appear to be very similar to the papain digest pieces I and II. The reduction breaks only one disulphide bond (168) and is partly reversible (165). This reversibility has been used to combine fragments from different antibodies to produce 100,000 molecular weight material which contains unrelated combining sites on the same molecule [Nisonoff &

Rivers (171)]. Study of such artificial heterologating antibodies may be of value in establishing whether any similar antibodies occur naturally. Piece III is destroyed under conditions of peptic digestion. In the papain digestion, cysteine is present as an enzymic activator, so it is probable that a disulphide bond is also broken under these conditions, though this was not recognized at the time. Clear confirmation that the papain digestion follows this course has now been obtained, using papain rendered insoluble by coupling to *p*-amino-phenylalanine-leucine copolymer [Cebra *et al.* (45)]. The insoluble enzyme was activated, washed free of cysteine, allowed to hydrolyze γ -globulin until 3 to 5 peptide bonds had broken, and then removed. There was no change in molecular weight, and the antibody would precipitate with the appropriate antigen as it did before hydrolysis. However, addition of cysteine split the molecule to give pieces I, II, and III. If cysteine was added to a specific precipitate of partially hydrolyzed antibody, the precipitate dissolved to give III, together with I and II, bound to the antigen. It is clear that I and II are joined through a disulphide bond, and perhaps III is bound, similarly, to either or both.

Some of the biological properties of the antibodies have been studied, and they show an interesting distribution among the different pieces. For example, the ability of an antibody to cross the foetal membranes has been shown to be lost in several species after partial digestion with pepsin [Hartley (109); Brambell *et al.* (27)]. This suggested that the part lost might have some special property that enabled the transfer to occur. An investigation of the rates of transmission of the three pieces showed that III would cross into the yolk sac almost as rapidly as whole γ -globulin, while the passage of I and II was very much slower. This suggested that on III there is indeed some kind of structural feature responsible for this activity [Brambell *et al.* (28)]. Passive cutaneous anaphylaxis can be produced in guinea pig skin by the intracutaneous injection of rabbit antiserum followed by intravenous injection of the antigen. A papain digest of rabbit antibody will not give this reaction. If rabbit γ -globulin is used as antigen and injected intracutaneously in reversed passive cutaneous anaphylaxis, a positive reaction will be produced by subsequent injection of horse antirabbit γ -globulin. If the pieces are used in this reverse test as antigens, then a positive reaction is given by III but not by I and II [Ovary & Karush (180)]. These results are interpreted to mean that for the forward reaction it is necessary for a molecule both to attach to a site in the skin and to combine with antigen. None of the pieces have both properties. For the reverse reaction, power to attach to the skin and combine with antibody is necessary. With suitable antisera, all pieces can be shown to be able to combine with antibody, but only III can attach to the skin. Hence some sort of skin-attaching site is present on III. Relevant to this is the observation that macroglobulin (19S) antibodies cannot produce a passive cutaneous anaphylactic reaction, although they are able to combine with antigen *in vitro* and to inhibit (by competing for antigen) the forward reac-

tion, using antibodies of the same specificity but with a molecular weight of 170,000 [Ovary *et al.* (179)]. It is inferred that the skin-attaching site on the part of the molecule equivalent to piece III is missing or is blocked in antibodies of large molecular weight. This raises the possibility that the failure of a large molecular weight antibody to pass from mother to foetus [see Kunkel (136)] may not be an effect of size, but may be due to blocking of the membrane-transmission site present in the same part of the molecule as the skin-attaching site. The antigenic sites responsible for the allotype reaction appear to be on pieces I and II, at least in certain cases [Kelus *et al.* (131)]. This is in contrast to antigenic sites which come into play when rabbit γ -globulin is injected into other species. These sites may be on all three pieces but are predominantly on III.

Papain digestion of γ -globulin from species other than rabbit causes a similar split to produce pieces with a sedimentation coefficient of 3.5S. The rate of digestion and the production of small peptides varies with the species used [Hsiao & Putnam (119)]. The yields of nondialyzable substances during enzymic digestion may be influenced by the method of γ -globulin preparation. The very limited hydrolysis is due to the inaccessibility of the many potentially hydrolyzable bonds and a slight alteration of the configuration during preparation could greatly increase the number of bonds broken. Thus, γ -globulin prepared by cold ethanol methods might be more vulnerable than material prepared by using ion-exchange cellulose. Several laboratories have investigated the products of papain digestion of human γ -globulin [Edelman *et al.* (67); Stiehm *et al.* (232); Franklin *et al.* (85)]. Since different methods of fractionation and different nomenclatures for the products have been used comparison of the results is not easy. However, there appear to be two main products of which one is present in approximately twice the amount of the other, though some variability has been noted. The variation may depend on the preparation of the γ -globulin or on differences in times of digestion, as some change of relative yields with time has been observed [Hsiao & Putnam (119)]. In the one case where it was tested [Franklin (85)], most of the antibody activity was associated with the component present in the higher amount.

Both fractions of the human γ -globulin digest react with rabbit antihuman γ -globulin serum. Edelman *et al.* (67) noted that with some antisera the undigested molecule gave a double line in immunoelectrophoresis and that the S digest fraction (electrophoretically slow) was antigenically related to the inner line while the F (faster) digest fraction was related to the outer line. This could be interpreted to mean that there are two antigenically distinct types of γ -globulin, one giving rise to fraction S and one giving rise to fraction F. A similar phenomenon is observed with mouse myeloma γ -globulin which gives a double line with rabbit antimyeloma γ -globulin sera and also gives similar S and F components after papain hydrolysis [Askonas & Fahey (13)]. Again components S and F are antigenically distinct, and the antibody

in rabbit antisera reacting with either could be adsorbed out with the appropriate component [Askonas & Humphrey (12)]. Such adsorbed sera, however, precipitated all the undigested γ -globulin rather than just a part, as would be expected if there were two antigenically distinct types present originally. The authors therefore suggest that the antigenic groupings present on both fractions S and F are contained in every molecule of γ -globulin, but that they may be available to the antibodies to differing extents. This could give rise to a double line in the reaction of whole γ -globulin with the antisera.

When the papain digest of γ -globulin from immune mouse serum was examined, it was found that the antibody activity was associated with the S fraction which was present in about twice the amount of the F fraction [Fahey & Askonas (77)]. Rabbit, human, and mouse γ -globulin are split by papain into pieces approximately one-third the original size. In the rabbit, three fractions can be separated by chromatography, and two are found to contain antibody-combining sites. Using similar methods, only two main fractions are obtained in the mouse and human γ -globulins, and one of these, the S fraction, is present in about twice the yield of the other; the component present in larger amount can be fractionated further. Since the antibody activity is associated with the S fraction, it seems likely, at present, that this larger component is the equivalent of pieces I and II in the rabbit, suggesting a basically similar structure in the antibody of all three species, as would indeed be expected.

Immunoelectrophoresis of γ -globulin prepared from stored human serum or plasma has shown that hydrolysis may occur during storage and that the products are similar to those produced by trypsin, plasmin, and papain [Skvafil (218); Augustin & Hayward (15)]. Relatively low-molecular-weight proteins which react with antihuman γ -globulin serum have also been found in human urine [Franklin (84); Berggård (20, 21); Webb *et al.* (244)]. Berggård found this protein to be present also in the plasma and Franěk *et al.* (83) reported a somewhat similar protein in the plasma of newly born pigs.

Splitting of γ -globulin by reducing agents and urea. In 1959 Edelman (66) reported briefly that normal human γ -globulin and macroglobulin could be dissociated to lower molecular weight by reduction in the presence of urea. As two or three peptide chains had been deduced to be present in the molecule from N-terminal amino acid assay [Putnam (194)], this was not unexpected. However, in a full report [Edelman & Poulik (68)] and from other work [Franěk (82); Ramel *et al.* (196)] it appears that following such treatment in all the species examined—human, rabbit, pig, cow, and horse—there is a substantial fall in molecular weight to the order of 40,000 to 50,000 suggesting the presence of at least three peptide chains in the γ -globulin of these species. This is not in agreement with N-terminal amino acid data. In the rabbit, only one N-terminal alanine residue, together with smaller amounts of serine and aspartic acid, can be detected; in horse and cow γ -

globulins some four or five N-terminal amino acids are present but together add up to less than 1 mole per mole of γ -globulin; and in pig γ -globulin three to four molecules of N-terminal amino acid are present [see Porter (189)]. Attempts have been made to reveal unreactive N-terminal amino acids in rabbit γ -globulin by exposure to strong denaturing agents [Porter (185); Cebra *et al.* (45)] and reducing agents [Porter (188)] but without success. Assay of the N-terminal amino acids of human and pig γ -globulin using the phenyl isocyanate method [Eriksson & Sjöquist (73)] gave results very similar to those found with the fluorodinitrobenzene method. It seems clear that γ -globulin must contain at least three polypeptide chains but that some of the N-terminal amino acids are blocked or poorly reactive under the conditions so far used.

Conditions chosen for reduction of the γ -globulin have been 0.1 *M* mercaptoethanol in 6 *M* solution of urea followed by blocking with iodoacetamide [Edelman & Poulik (68)] or S sulphonation in 8 *M* solution of urea or 0.1 *M* sodium dodecylsulphate [Franěk (82)]. In both cases the products are insoluble in water and the fractionation and molecular weight estimations have been carried out in 6 *M* urea. Attempts to fractionate the reduction products were made, using chromatography on ion-exchange cellulose and starch-gel electrophoresis always in 6 *M* solution of urea [Edelman & Poulik (68)]. Though recoveries in chromatography were low (50 to 60 per cent) fractionation was achieved and could be partly correlated with the behaviour on starch-gel electrophoresis. The electrophoresis picture was more complex than the three or four bands of equal strength which would have been expected if all the γ -globulin molecules were identical and consisted of three or four peptide chains of similar size. Though it is not yet possible to form a clear idea of the general structure of γ -globulin, it seems certain from this work and the enzymic digestion, that γ -globulin from all species has a similar structure and that the big differences suggested by N-terminal amino acid assay are not correct. The similarity in size of the products obtained, whether by reduction or by papain digestion, is striking, and correlation of the results from the two methods should give a clearer picture of the over-all structure.

Allotypes.—In 1956, Oudin (174, 175) reported that rabbit γ -globulin exists in several antigenically distinct forms which he termed "allotypes." They were found by examining the antisera produced after injecting specific precipitates of rabbit antibody into other rabbits. In certain cases the recipient rabbit responded by producing antibody which could be shown by gel diffusion methods to react with γ -globulin from the donor rabbit. These antigenic differences are genetically controlled. A full study of this phenomenon has now been published [Oudin (176, 177)] and the following conclusions drawn: (a) There is an antigenic species specificity (isotypic) which is common to γ -globulin from all rabbits. (b) In addition, there are at least seven individual antigenic variants of which two or more are present in the γ -

globulin of all animals. (c) For the preparation of antisera, the antigenic types of the injected γ -globulin must be absent from the γ -globulin of the recipient. (d) If two or more allotypes are present in one individual it is probable, though not certain, that the different allotypes are present in different molecules. (e) If guinea pig antisera are prepared against γ -globulin of different allotypes, no differences are shown, i.e., the guinea pig reacts only to the isotypic sites. (f) In newly born rabbits, γ -globulins with allotypes of the mother predominate and gradually change as the content of the animal's own γ -globulin rises.

Considerable progress has been made in working out the genetic relationship of these antigenic variants, and similar studies have been reported by Dray & Young (56, 57, 58) and by Dubiski *et al.* (61, 62, 63). At present, the use of different nomenclature by the three laboratories makes comparison of the results difficult, and it is hoped that a common scheme will be adopted very soon.

A related variation in antigenic structure of human γ -globulin has been shown by immunizing rhesus monkeys with γ -globulin prepared by ion-exchange cellulose chromatography. Immunoelectrophoresis of human serum using monkey antiserum showed that there were at least three antigenically distinct types in pooled human γ -globulin [Dray (55)]. Another phenomenon observed in human serum, the Gm groups, may be related to the antigenic variation shown by the allotypes. The test system is measurement of inhibition by the γ -globulin of the agglutination of red cells (coated with incomplete Rh antibody) by rheumatoid arthritis serum [Grubb & Laurell (105); Grubb (103, 104)]. It is possible that this rheumatoid factor is some kind of autoantibody to γ -globulin, but the complexity of the system is such that it is not certain that a genuine antibody-antigen interaction is occurring. Nevertheless, four types of human γ -globulin have been distinguished in this way and are again genetically controlled [see also Fudenberg & Kunkel (88)]. Rabbit γ -globulin has some inhibitory activity, though less than human γ -globulin in this system, and this activity appears to be associated with the papain digest piece III [Goodman (94)].

Nonprecipitating and S_{19} antibodies.—In 1940, when immunizing a horse with ovalbumin, Pappenheimer (181) observed that in the early bleeding a considerable proportion of the antibody was nonprecipitating. This phenomenon has been observed in other species. In rabbits, if very poor antigens are used such as insulin [Berson & Yalow (22); Grodsky & Forsham (101)] and glucagon [Unger *et al.* (236, 237)], only nonprecipitating antibodies are formed, however long the immunization is continued. When gelatin derivatives are injected into rabbits, mixtures of precipitating and nonprecipitating antibodies are produced (7, 208). It is possible that nonprecipitating antibodies are an intermediary product formed when any animal is immunized, but that with a good antigen this stage is so short that it is not recognized. Alternatively, the antibodies may be bivalent but can react with only one

site on the antigen. That is, the antigen is monovalent with respect to these sera, and hence when added to them does not cause a precipitate to form.

The relation of 19S antibodies to 7S antibodies is not clear. Petermann & Pappenheimer showed that treatment of both 7S horse antidipteria toxin (183) and 19S horse antipneumococcus polysaccharide (184) with pepsin gave a product with a molecular weight of about 100,000 which would still give a precipitate with the appropriate antigen. From this it might be concluded that their structures must be basically similar, and that the high molecular weight material is some type of polymer of the normal γ -globulin. However, there are differences in the biological properties of the two types of molecule from human sera, such as antigenic specificity [Franklin (86); Ovary *et al.* (179); see also Kunkel (136)], attachment to skin [Ovary *et al.* (179)], passage from mother to foetus [Kunkel (136)], and turnover rate [Cohen & Freeman (49)]. The antigenic differences persist even when the S_{19} antibodies are dissociated to give 7S molecules with thiol. In some cases this dissociation destroys the antibody activity [Fudenberg & Kunkel (87); Chan & Deutsch (46)]. These results are not in accord with any simple polymerization theory.

A novel finding of considerable interest is the observation of Smith (220) that if prematurely born infants are immunized with bacterial antigens, such as *Salmonella typhi*, there is a significant antibody response and the antibodies are macroglobulins. The molecular weight decreases on the addition of thiol, but there is no loss of antibody activity. The implication of this finding is that S_{19} antibody may be synthesized in a different cell type to that making S_7 antibody. However, the rheumatoid factor appears to be made in plasma cells [Mellors *et al.* (156, 157)]. A similar investigation of antibody production in newborn piglets failed to show any kind of antibody formation [Franěk *et al.* (83)]. Further evidence has been given to show that nonprecipitating antibodies of human sera responsible for immediate type allergy (the reagins) are in the γ_1 -fraction and have a sedimentation coefficient of $S=7$ [Stanworth (225)]. There may, however, be other types of antibody also responsible for this type of allergic reaction [Augustin & Hayward (16); Cooke *et al.* (50); Gyenes *et al.* (108)].

Turk (235) has reported briefly that delayed type sensitivity to picryl chloride, in guinea pigs, can be passively transferred to normal animals by injection of a suspension of broken lymph node or spleen cells from a sensitized animal. Lawrence (145, 146) has demonstrated this phenomenon in humans, but no success had been achieved previously in guinea pigs. Turk was able to transfer this activity with either cell sap or microsomes from cells broken either by freezing and thawing, sonic disruption, or homogenization. The activity in the cell sap survived dialysis and preliminary chromatography on ion-exchange cellulose. This work may well lead to a clarification of the apparent difference between so-called cellular immunity and humoral immunity.

Blocking of polar groups in antibodies.—Attempts to determine the chem-

ical features of antibody-combining sites by blocking different polar groups have been continued over many years, and the difficulties of interpretation of the results are such that convincing conclusions are difficult to obtain. However, recent work by Pressman and colleagues has brought strong evidence from acetylation and iodination experiments that the tyrosine residues are of importance in several rabbit antibodies [Pressman *et al.* (193); Johnson *et al.* (123)]. Earlier acetylation experiments showed that most of the amino groups could be blocked without altering the binding of hapten to antihapten antibody, although precipitating power was lost (167). Also acetylation of 96 per cent of NH_2 groups of antbovine serum albumin resulted in loss of precipitating power, but complexes of bovine serum albumen and antibody were demonstrated by electrophoresis and ultracentrifuge studies [Pressman *et al.* (192)]. Esterification of carboxyl groups reduced the affinity of antibodies for the corresponding hapten if the latter was basic, but not if it was acidic [Grossberg & Pressman (102)]. This suggested that electrostatic forces may be important in antibody-antigen reaction and further evidence in support of this view came from studying specific anion effects [Pressman *et al.* (191)].

Koshland, Engelberger & Koshland (135) have proposed that these methods of blocking active sites of antibodies could be taken further to get specific labelling in the following manner: (a) block all reactive groups of the antibody in the presence of hapten; (b) remove the hapten. (c) block the groups protected by hapten with a radioactive reagent. In this way, specific labelling of the site should occur, and identification of the peptides protected by the hapten and presumably associated with the antibody site should be possible. Iodine was chosen as the reagent and difficulties arose because the haptens did not protect the antibody activity under conditions where maximum iodination took place. Hence, more gentle conditions had to be used and further iodination of nonspecific sites with I^{131} occurred when this reagent was used in the absence of hapten. This, together with difficulties in obtaining adequate fractionation of peptides from a molecule as large as γ -globulin, offers formidable problems.

ANTIGENS

Much work is devoted to the solution of the complementary problems of the structural basis of antigenic activity. Here there are two problems: (a) what properties enable a substance to initiate an antibody response? (b) to which structural features of the antigen are the antibody-combining sites directed? To the latter question, a plausible answer has been suggested by Burnet (37). He postulated that if a protein such as bovine serum albumin were injected into a rabbit, then antibodies would be formed which were directed to those parts of the structure not identical with those of rabbit serum albumin or any other rabbit protein. Cinader (47) has discussed this further. With the rapid growth in our knowledge of protein chemistry, it may soon be possible to test this hypothesis.

The structure of different types of antigen has been studied by blocking groups or by attempting to characterize smaller active pieces. Another approach has been to add on compounds of a known structure and study their influence on antigenicity and specificity. This has been taken to its logical conclusion recently in using entirely synthetic antigens.

Synthetic methods.—The synthetic methods of Landsteiner established the chemical basis of antigenic specificity, and the potential contribution of such methods to our knowledge of the structural basis of antigenicity was shown when Harington & collaborators (48) greatly increased the antigenic power of insulin and gelatin by the addition of tyrosine. With the introduction of amino acid polymers, the possibilities of these methods have greatly increased.

The influence of substituents on specificity and the inhibitory power of the corresponding haptens has, in recent years, been directed largely toward trying to get more information about the nature and size of the antibody-combining site. Work by Beiser *et al.* (19) on inhibiting the precipitation of rabbit antibodies to the β -D-galacto-pyranosyl structure by related compounds has led to the conclusion that the specificity of the antibody is directed only towards the galactosido-phenylazo fragment and does not include the attached amino acid. The size of antibody site deduced from this is about the same as that calculated by Karush (128) using antihapten antibodies, and is about half that derived by Kabat (124) from the inhibitory study using polyglucose units in the dextran-human antidextran system. No reference is made to the very low inhibitory power of these haptens which are claimed to form a complete antigenic site. It appears that in the work of Beiser *et al.* (19) a molar ratio of 1000 hapten/antigen is required for 50 per cent inhibition using the most effective hapten. Ratios of the same order or greater were found in the work of Karush & Kabat (124, 128), and again in the inhibition studies by peptides, of the precipitation of silk fibroin and rabbit antiserum [Cebra (44)]. If these compounds were the complete antigenic sites, it would be expected that their affinity for the antibody would be as great as that of the same site in the full antigen, and the competition would be much more effective, as has indeed been found in work with digestion products of protein antigens. For example, a 12,000 molecular weight fragment of bovine serum albumin gives 50 per cent maximal inhibition at a molar ratio of 1.2/1 [Porter (186)] and a 7000 molecular weight fragment of human serum albumin is similarly effective at 1.5/1 [Press & Porter (190)]. In both cases, the native configuration of the polypeptide is essential for activity. Differences in inhibitory power of this order are difficult to reconcile, and suggest that the haptens may be only part of a much larger antigenic site. Further work will be required to determine how large this is, and indeed if precise limits do exist. However, caution is necessary before it is assumed that antibody sites are small, particularly when deductions from this are used in theories of antibody formation [Talmage (234); Haurowitz (110)].

In the second problem—the basis of antigenicity—most recent work has been concerned with the effectiveness of polyamino acids, either alone or bound to proteins. Stahmann & collaborators (33, 224) attached polypeptides of leucine, lysine, glutamic acid, or phenylalanine to bovine or rabbit serum albumin and found that on injection into rabbits, antibodies were found with specificity directed to the polymers, polymers and protein, and, in the case of bovine albumin, carrier protein alone. Sela & Arnon attached short chains of different amino acids to gelatin and tested their efficiency in raising the antigenic power of this very poor antigen. Tyrosine, tryptophane, and phenylalanine were very efficient; cysteine less so; and glutamic acid, lysine, alanine, and serine quite ineffective (208). Mixed polymers of glutamic acid and tyrosine were better than polytyrosine (7), and the specificity of the antibodies was directed to the substituent polymers. Polycyclohexanyl gelatin was also antigenic, and this led to the suggestion that the rigidity of structure caused by addition of aromatic groups, and to a lesser extent by addition of disulphide bridges, might be an important factor in determining antigenic power (209).

Several attempts have been made to induce antibody formation by the injection of amino acid polymers. Maurer (153) was unsuccessful using polyglutamic acid but did obtain evidence of antibody formation, detectable by passive cutaneous anaphylaxis, with a mixed polymer of L-glutamic acid and L-lysine of molecular weight about 40,000 [Maurer *et al.* (154)]. Stahmann & collaborators (34, 35) used a variety of polymers and copolymers but the results were rather confusing. Rabbits, chickens, guinea pigs, and mice were injected but only rabbits responded. Unusual antibodies were found which would not precipitate with the homologous antigen (polyglutamic acid) but would precipitate with the polymer coupled to bovine serum albumin and even with the unsubstituted native albumin. Clearer results were obtained by Gill & Doty (92) who injected rabbits with a mixed linear polymer containing glutamic acid, lysine, and tyrosine in the ratio 2.8:2.6:1. The sera contained as much as 4 mg antibody/ml, but again there were several unusual features. All reactions were carried out at pH 10.5 (because of the insolubility of the polymer near neutrality) and the precipitin curves were stated to show a series of optima. In the gel-diffusion tests at pH 10.5, zones of precipitation were broad. This and the low solubility of the polymer suggest some kind of artefact, but control sera under the same conditions did not show any precipitation.

The only example of antibody production similar to that stimulated by natural antigen was obtained by Sela & Arnon (210). These authors used a more complex polymer with a backbone of alanine and lysine residues. Short chains of glutamic acid and tyrosine were then condensed onto the ϵ -amino groups of lysine. The molecular weight was 180,000 and the composition was DL-alanine, 67.5 per cent; L-lysine, 7.0 per cent; L-tyrosine, 9.9 per cent; and L-glutamic acid, 15.3 per cent. When this was injected into rabbits with adju-

vant, antisera were obtained which contained about $\frac{1}{2}$ mg antibody/ml and gave typical rabbit precipitation curves with a single optimum. The antisera were specific for the homologous antigen and showed a cross reaction only with a polymer of tyrosine and glutamic acid condensed on to gelatin. This work seems to open the way to a systematic examination of all the variables which may play a part in endowing a compound with antigenic power.

It now seems clear that the sera of some patients suffering from disseminated lupus erythematosus contain antibodies which are specific for DNA [Seligmann (211); Deicher *et al.* (53)] and the antigenic sites appear to be common to all types of DNA [see also Meischer *et al.* (158)]. Injection of disrupted phage into rabbits stimulates the production of antibodies specific for phage DNA [Levine *et al.* (148)]. It is still probable that DNA is not antigenic but is functioning as a hapten much as the pneumococcal polysaccharides behave in rabbits. The species variation in antigenicity of these large molecules is relevant to the general problem of antigenicity discussed above.

Analytical methods.—It was recognized many years ago from cross-reaction experiments that antibodies produced by an animal against a single antigen are complex. Thus, in rabbit antisera to hen ovalbumin some antibodies will precipitate with the ovalbumin of duck or of turkey and some only with the homologous protein. This might have arisen because of variation in the closeness of fit of sites from different antibodies, or because some antibodies were directed to antigenic sites common to the different ovalbumins and some to sites unique to the homologous protein. The multiplicity of antigenic sites rather favoured the second alternative and this has been discussed, with reference to antiviral antibodies, by Rappaport (197).

Direct attempts have now been made to split off and characterize the individual antigenic sites. The work was initiated, as was much else in this field, by Landsteiner (137), who found that a partial acid hydrolysis of silk fibroin retained the power to inhibit the precipitation of the whole antigen with its rabbit antisera. Little fractionation of peptides was possible at that time, but the work has now been continued by Cebra (42, 43, 44), working in the same laboratory. A chymotryptic digest of silk fibroin was fractionated, using ion-exchange and partition chromatography, zone electrophoresis, and filtration through Sephadex—all methods developed since Landsteiner's work. Several tetra-, octa-, and dodecapeptides were isolated, analyzed, and the partial amino acid sequence determined. Fifty per cent inhibition of maximal precipitation was achieved with about 1000/1 peptide/silk fibroin ratio with the dodecapeptides, and a bigger ratio for the smaller peptides. As would be expected from a chymotryptic digest, tyrosine was C-terminal in many of the peptides, and its removal by carboxypeptidase caused a sharp drop in inhibitory power although some activity remained.

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trypsin [Lapresle *et al.* (140, 141)]. With the first two enzymes, immunoelectrophoresis showed the presence of three distinct antigenic fragments, and with a tryptic digest, the presence of two. Lapresle & Webb (142) have described the purification, from a catheptic digest, of a fragment which will precipitate only 5 per cent of the antibody from the antisera and which is an effective inhibitor. Fractionation by zone electrophoresis and chromatography on diethylaminoethyl cellulose gave material with a sedimentation coefficient $S_{20} = 1.36$. The molecular weight was stated to be in the range 12,000 to 20,000. Calculations from their data suggests that 50 per cent maximal inhibition was given with an inhibitor/antigen molar ratio of between 1 and 2, similar to that found for a 12,000 molecular weight fragment from bovine serum albumin [Porter (186)]. Lapresle (138) has also used these partial hydrolysis products to study the complexity of rabbit antihuman albumin sera. Similar studies using ovalbumin as antigen have been commenced by Kaminski (126). Richard *et al.* (198) separated a variety of small polypeptides from a chymotryptic digest of bovine serum albumin and found that some would give lines with the antisera in agar gel plates but more detailed characterization of the peptides will be required to interpret these results. Morton & Deutsch (160) partially purified, from a papain digest of ovomucoid, a peptide of molecular weight about 6,500 which inhibited combination of the whole antigen with its rabbit antisera. Partial hydrolysis studies have also been reported using casein (89) as well as fibrinogen (173, 203) as antigens. The influence of physical dissociation of tobacco mosaic virus on its antigenic specificity was studied by Kleczkowski (134).

If this type of work could be carried out with a protein of known chemical structure, the chances of really defining the antigenic sites would be greatly increased, and several papers have now appeared in which ribonuclease has been used as antigen. Brown *et al.* (30) found that reduction of the disulphide bonds destroyed all antigenic specificity, thus confirming the importance of the steric structure. Partial deamination [Van Vunakis *et al.* (238)] reduced the precipitating power somewhat variably, depending on the antisera used to test, and the evidence suggested that the most important group being affected was the N-terminal lysine. Singer & Richards (216) compared the precipitating power of ribonuclease before and after hydrolysis with subtilisin. This enzyme hydrolyzes only one bond in the native ribonuclease—20 residues from the C-terminal end. The 20 residue peptide (S peptide) remains bound to the protein (S protein) by noncovalent linkage. No loss of enzymic activity occurs, but there is a decrease in precipitating power that suggests a change in tertiary structure not affecting the enzymic active centre. If the S protein and S peptide are separated, the peptide has no affinity for the antibody and the S protein will precipitate less than half the antibody precipitated before separation. The authors suggest that this may be the result of configurational changes of the separated protein and peptide or caused by their both playing a part in forming the same antigenic site. Brown & Delaney (32) have reported briefly on the isolation of peptides from a tryptic

digest of oxidized ribonuclease which will inhibit the reaction of the oxidized enzyme with its antisera.

When rabbits were immunized with bovine ribonuclease and tested with the enzymes prepared from cow, sheep, and pig, Brown *et al.* (31) found that the sheep enzyme gave partial cross-reaction and the pig gave none after one immunization course. After a second course, the sheep enzyme gave complete cross reaction and the pig, a partial reaction. The chemical difference between the sheep and cow enzymes is believed to lie in only three amino acids: one a replacement of serine for threonine in residue 3, another a lysine for glutamine in residue 37, and a third not yet defined [Anfinsen *et al.* (4, 5)]. The change of a neutral for a basic residue could alter the configuration considerably and may be a guide to the position of an antigenic site. The pig enzyme, which as expected shows less cross-reaction, appeared, from a preliminary peptide pattern, to be identical with the cow enzyme [Katz *et al.* (130)], but it would be expected that further work will show significant differences.

A distinction between enzymic and antigenic sites was shown in alkaline phosphatase [Schlamowitz (205)]. Reduction with cysteine destroys the catalytic but not the precipitating activity of the enzyme, while acetylation stops precipitation with little effect on enzymic activity. Richmond (199) grew *Bacillus cereus* in a medium containing *p*-fluorophenyl alanine, prepared exopenicillinase and found that 5 to 6 of the 7 phenyl alanine residues were replaced by the fluoro derivative. Both the enzymic activity and antigenic specificity were changed. As the author points out, the purified enzyme was probably a mixture of molecules in which the abnormal amino acid was present in different portions. If such a mixture could be fractionated and the place of substitution identified, it could offer a novel method for studying the nature of the antigenic sites of the enzyme.

Studies of partial hydrolysis have led Ishizaka *et al.* (121) to consider whether proteins are broken down *in vivo* to give rise to antigenic fragments with specific sites not present in the whole molecule. To test this, rabbits were immunized in several ways with crystalline bovine serum albumin, the antisera absorbed with this protein and then tested for reaction with a peptic digest of the protein. In this way evidence was obtained suggesting that antibodies were sometimes present which could not precipitate with the whole molecule, but which would give a ring test and passive cutaneous anaphylaxis with the peptic digest. It is probable that the crystalline albumin used for immunization would contain other proteins. These might be antigenic and also more resistant to peptic hydrolysis than the albumin and thus could give rise to the results obtained. This possibility is unlikely but should be worth testing. Earlier, Lapresle & Durieux (139) studied the antisera that had been prepared by injecting rabbits with a spleen protease digest of serum albumin. It was their intention to determine whether such degradation occurred naturally prior to antibody formation, but they were unable to arrive at any definite conclusions.

Immunochemical methods are used increasingly in the characterization

of protein and other antigens. For example, the relationship of various species of potato has been studied by cross reaction with antisera in gel medium and results were obtained which agree well with classical taxonomic methods [Gell *et al.* (90)]. Oudin (178) has identified some 26 different antigens in human serum by related methods. Cross reaction of pituitary growth hormones of different species have been found to be extensive, and immunochemical assays have been developed [Li *et al.* (149, 161); Boucher (23)]. In the fractionation of eye lens protein, the precipitation of the fractions was followed by reaction with antisera in gel medium [Manski *et al.* (151)].

ANTIBODY-ANTIGEN REACTION

A new technique for the study of antibody-antigen interaction, the quenching of fluorescence, has been introduced by Velick *et al.* (240). Though applicable to only a limited range of antigens and haptens, it has many advantages. Proteins absorb light of wavelength 260 to 290 $m\mu$ because of their content of aromatic amino acids. The energy absorbed by tyrosine is largely dissipated, but of that absorbed by tryptophane about 20 per cent is emitted as fluorescence, with a maximum at 350 $m\mu$. If a hapten such as dinitrophenol, which absorbs at this wave length, is chosen, then on combination of antibody and hapten the fluorescence emitted by the antibody is quenched. Measurement of the quenching gives a quantitative estimation of the combination. This technique was used to study the interaction of purified antibody as well as that of the pieces produced by papain digestion with the haptens dinitrophenol and azobenzene arsonate. The speed and simplicity of the measurements enabled the authors to study a wide range of conditions, and the following conclusions were drawn: (a) hapten binding was remarkably constant over a very wide pH range and decreased markedly only below pH 1.5 and above pH 10.5; (b) a temperature range of 4° to 60° C could be used, as no irreversible changes occurred at the higher temperatures during the few minutes needed for measurement (with the dinitrophenol hapten $\Delta F^\circ = 11.3$ kcal./mole⁻¹ and $\Delta H^\circ = 8.6$ kcal./mole⁻¹); (c) papain proteolysis pieces I and II were shown to contain one binding site per mole and III had no affinity for the hapten; (d) there appeared to be a much higher degree of homogeneity of hapten binding sites than that found by Karush (127, 128) using equilibrium methods, but this may be due to the selective nature of the method used to purify the antibody; (e) the efficiency of the quenching of fluorescence of whole antibody by hapten suggests that there may be some natural division of the molecule that corresponds to the breaks caused by papain digestion.

Further thermodynamic data for antigen antibody reactions have been given by Pepe & Singer (182) and Stelos *et al.* (228) using established methods of electrophoresis, sedimentation, and light scattering. The results of the first two methods were analyzed, using the Gilbert-Jenkins theory (91), and have been found to differ from the true values by factors little greater than

the experimental error [Singer *et al.* (215)]. Thermodynamic functions of different systems are listed in Table I.

Apart from the values for ϵ -N-dinitrophenyl-lysine hapten, the standard free energy appears to be independent of the antigen valency and structure, but there is about 3 kcal difference between a protein antigen or a hapten bound to protein and a free hapten. (It is interesting to note that the ΔF° for

TABLE I
THERMODYNAMIC FUNCTIONS OF RABBIT ANTIBODY AND
DIFFERENT ANTIGEN SYSTEMS

Antigen	ΔF° kcal/mole	ΔH° kcal/mole	ΔS° eu	Reference
Benzene arsonic acid hapten (1 valency)	-7.7	-	-	(70)
ϵ -N-dinitrophenyl-lysine hapten (1 valency)	-11.3 \pm 0.3	-8.6	9	(240)
Terephthalanilide diarsonic acid hapten (2 valencies)	-7.7	1 \pm 2	23 \pm 7	(72)
Phenyl-[<i>p</i> -(<i>p</i> -dimethylamino- benzeneazo)-benzoxylamino]- acetate (1 valency)	-7.3 \pm 0.5	-7.2	0	(127)
Benzene arsonic acid-bovine serum albumen (multivalent)	-4.5	0 \pm 2	17 \pm 7	(17)
Bovine serum albumen-S- benzene arsonic acid (1 valency)	-5.0	-	-	(182)
Bovine ribonuclease (approx. 4 valencies)	-4.5 \pm 0.2	-	-	(228)
Bovine serum albumin (approx. 7 valencies)	-4.9	0 \pm 2	20 \pm 8	(214)
Ovalbumin (approx. 5 valencies)	-5.1	0 \pm 2	20 \pm 8	(213)
[Insulin self-association	-5.2	-7.7	-9	(54)

the self association of insulin molecules is close to that for the protein antigen-antibody interaction.) This difference in ΔF° is probably entropic and associated with steric factors [Pepe & Singer (182)]. The exceptionally low dissociation constant and the stability over a wide pH range of the ϵ -N-dinitrophenyl-lysine hapten-antibody complex show this system to have what are, at present, unique properties. The high value of ΔF° appears to be contributed to both by enthalpy [c.f. dye hapten of Karush (127)] and entropy factors. Further interpretation of these data will require more information on the interaction of solvent with both protein and hapten molecules.

Another method of studying antibody-antigen interaction has been reported briefly by Dandliker & Feigen (51, 52). Following a suggestion made earlier by Weber (246), they have applied the polarization of fluorescence technique to this problem and have followed the reaction of fluorescein-ovalbumin with the rabbit antihapten antibody. The intensity and polarization of the fluorescent light are measured for successive additions of fluorescent coupled antigen using, in the region of equivalence, dilutions sufficiently high to delay flocculation. The data given suggest that the method can give a quantitative measure of the interaction, and that it may also be applicable to the more general case of the interaction of fluorescein-labelled ovalbumin with antiovalbumin. This may be a method of particular value in following the reaction of non-flocculating systems.

Still another method of studying antibody-antigen reaction has been proposed by Ålbertsson & Philipson (3). In the two immiscible phases produced by water, dextran, and polyethylene glycol or methyl cellulose, both phases contain more than 95 per cent water. The antigen phycoerythrin partitions in favour of the top phase and γ -globulin in favour of the bottom phase. As soon as aggregates form they move to the interface, and the fall in content of phycoerythrin in the top phase gives a measure of the reaction rate. The reaction can be studied over a much wider antigen/antibody ratio than that in which precipitation occurs.

The effect of dioxane on the precipitin reaction has been studied by Grant (98, 99) and of alkali chlorides by Edelman & Bryan (69).

MECHANISM OF ANTIBODY FORMATION

Full accounts of work in this field have appeared in reports of two recent meetings [Wolstenholme & O'Connor (247)]; Holub & Jaroskova (117)]; and a review, Stavitsky (226).

Nossal (172) has given an account of his experiments on antibody production by single cells from the lymphoid tissue of rabbits immunized with different motile strains of *Salmonella*. Antibody against flagellar antigens was estimated by the power to immobilize the bacteria, and of 347 antibody-producing cells examined, none were found to produce more than one type of antibody. However, Attardi *et al.* (14) have made a very careful study of antibody production by single cells from rabbits immunized with two antigenically unrelated types of phage. In a preliminary report, it was stated that 15 per cent of the antibody producing cells synthesized both types of antibody. The full details of this work are still not available, but the positive result seems convincing. A modification of the clonal selection theory of antibody production that would accommodate the evidence of Attardi *et al.* has been proposed by Lederberg (147).

The necessity for the persistence of antigen at sites of antibody synthesis has long been in dispute in theories of antibody formation. It remains an intractable problem, as it is probable that the levels of antigen necessary to

stimulate antibody production are below the limits of detection by present techniques. Richter & Haurowitz (200) demonstrated the presence of antibody by haemagglutination seven months after a single injection of protein antigens, while Hawkins & Haurowitz (111) could demonstrate persistence of intracellular material with antigenic specificity for about a week after injection. The antigens appear to be associated with the ribonucleic acid rich fraction of spleen cells. Ward (243) showed the presence of Rh antibodies in women as much as 12 years after the last pregnancy, i.e., 12 years after the last known contact with the antigen. The life-long persistence of antiviral antibodies, sometimes with no evidence of repeated contact, has often been referred to [Burnet & Fenner (38)], and Herriott (115) has now suggested that this may be related to the ability of virus to exist in an animal in an infective but nonantigenic form.

Persistence of antigen as some form of inducer necessary for the secondary response in antibody formation is suggested by experiments of Dresser (60). He injected spleen cells from a mouse, primarily stimulated with a protein antigen, into x-irradiated mice of the same strain. The secondary response on subsequent challenge was estimated by the elimination rate of I^{125} -labelled antigen [Dresser (59)]. With this method, he found that, although sufficient time (six weeks) was allowed for the injected cells to divide and recolonize the lymphoid tissue, the response was proportional to the size of the inoculum. When a constant number of cells was injected, the response decreased with increasing time between injection and challenge. There is no evidence to suggest that the injected cells are not dividing normally in the x-irradiated recipients. Hence it appears that the mechanism responsible for antibody synthesis is not being replicated and that some factor possibly derived from the antigen is diluted out as the cells divide.

In *in vitro* studies of antibody synthesis, the balance of evidence still suggests that a primary response can only be induced *in vivo* [Stavitsky (226)], though there are also claims to the contrary [McKenna & Stevens (163)]. Studies of antibody synthesis by cell suspensions from immunized animals continue [La Via *et al.* (143); Vaughan *et al.* (239); Dutton *et al.* (64); Bussard & Huynh (40)] but no clear success has been achieved so far with disrupted cells [Askonas (10)]. Kern *et al.* (132) and Askonas (8, 9) found that antibody activity is always associated with the microsomes of antibody-producing cells. The antibodies could be dissociated from the microsomes by a variety of nonspecific methods used to separate protein and nucleic acids [Askonas (9); Feldman *et al.* (79)]. The antibody is also displaced specifically by γ -globulin and papain digestion piece III [Kern *et al.* (133)]. The latter observation suggests some specific association, and the evidence is strong though not conclusive that antibody is synthesized by the microsome fraction. Release of antibody into the extracellular fluid is not due to lysis or cytoplasmic shedding, as antibody may be secreted under conditions where no release of a cell sap enzyme (aldolase) occurs. Antibodies

are released in the order in which they are formed, but the mechanism is still not clear [Helmreich *et al.* (113)].

An apparently related phenomenon concerns the presence of a cytophilic antibody fraction [Boyden & Sorkin (25); Sorkin *et al.* (223); Boyden & Sorkin (26)]. These authors claim that there is a distinct type of antibody which is specifically bound to cells and can be released by warming the cells to 56° C for 30 minutes. Their results appear to be at variance with those of other workers, and the position is not clear [Brocklehurst *et al.* (29)].

The influence of inhibitors on antibody production has been studied *in vitro* [Dutton *et al.* (65); La Via *et al.* (144)] and after injection of antigen and mature spleen cells into newborn rabbits [Sterzl (229, 230, 231)]. The increase in the rate of antibody synthesis observed in cultivated spleen cells is prevented by 5-bromouracil deoxyriboside, but this does not affect other metabolic functions [Dutton *et al.* (65)]. β -3-Thienylamine appears to inhibit the early stages of antibody formation [La Via *et al.* (144)] and 6-mercaptopurine is most effective in the inductive phase of antibody production [Sterzl (230)]. The latter observation was taken as additional evidence for the importance of DNA in the inductive phase. Schwartz & Dameshek (206, 207) reported that injection of 6-mercaptopurine with human serum albumin into a rabbit prevented formation of antibodies against the albumin. If albumin was injected some weeks later, there was still no immune response, i.e., a state of tolerance appeared to have been produced. This work has been repeated and partially confirmed by several workers [Robinson & Christian (201); Meeker *et al.* (155); Hoyer *et al.* (118)]. If the antigen was injected with adjuvants, together with the purine analogue, the immune response was delayed but not suppressed. If 6-mercaptopurine was injected when skin grafts were made, then increased time of survival of the grafts was observed.

Recent discussions of theories of antibody formation have been given by Boyden (24) and Sorkin (222). Szilard (233) has proposed a theory for the control of the formation of specific proteins and developed it to give a new theory of antibody formation. This theory has some relation to an earlier one of Burnet & Fenner (39) in that it suggests that antibodies are enzymes retaining affinity for substrates (haptens) but lacking catalytic power (Burnet and Fenner suggested antibodies were adaptive proteases able to hydrolyze any proteins met in foetal life, but which at birth lost catalytic but not combining activity). Szilard (233) postulates a mutational change to account for this loss of enzymic activity. In keeping with the clonal selection theory, the formation of antibodies specific for particular types of haptenic groups is under genetic control. Antigen initiates antibody production by removal of an enzyme which couples together the two essential parts of the repressor. Antibody molecules can themselves bind repressor molecules so that when once initiated they inactivate any remaining repressor molecules and keep the cell locked into a state of continued production of that antibody. When this occurs there is a reduction in the rate of production of other cellular proteins,

including a hypothetical enzyme S which inhibits cell division. In the secondary response the antigen enters, precipitates antibody present, and coprecipitates the enzyme S reducing its concentration to zero. Hence, rapid division of the antibody producing cells proceeds, giving rise to the secondary response of antibody production. These ideas are developed further to explain immune tolerance and other phenomena and lead to several predictions capable of experimental test. One of these predictions is that in a rabbit a good antigen such as bovine γ -globulin coupled to hapten, and a poor antigen such as rabbit albumin coupled to the same hapten may give a very different response in the primary injection, but should behave similarly in a secondary response.

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INSECT NUTRITION¹

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Most research on insect nutrition is on the dietetics and nutritional requirements of many insects, the relations between their requirements and metabolic processes, and the links between certain physiologic and ecologic phenomena. Levinson (1), Lipke & Fraenkel (2), and House (3) reviewed the subject in recent years. In addition, there are reviews of the subject concerning only amino acid and vitamin requirements (4), silkworms (5), phytophagous insects (6), parasitic insects (7), and nutritional pathology (8). Related to these are discussions on digestion (9), symbiosis (10, 11), insect biochemistry (12, 13, 14), nutrition and humoral control of reproduction (15), nutrition and insect resistance to chemicals (16), and the reaction of pests to host-plant nutrition (17). In the present paper, emphasis is on our understanding of nutritional requirements and on our insight into insect metabolism arising from nutritional research, though some other aspects are briefly discussed.

Some terms may be clarified as follows: "nutritional requirements" are the chemical factors of ingested food essential for normal metabolism and development of the insect; "chemical feeding requirements" and "physical feeding requirements" are, respectively, the chemical and physical factors important to normal feeding behavior, as Beck (18) defined them. A "chemically defined" diet in this paper embraces what Dougherty (19) termed either "holidic," pertaining to media whose intended constituents, other than purified inert materials, have exactly known chemical structure before compounding, or "meridic," pertaining to media composed of a holidic base to which is added at least one substance or preparation of unknown structure or of uncertain purity. "Axenic" refers to the rearing of only one species on a nonliving medium (19). Generic and specific names of insects are those in current usage.

TECHNIQUE AND EXPERIMENTEE

Our limited knowledge of the content of natural foodstuff reduces its value in precise experiments. Techniques on excised plants, for example, may have serious inherent sources of error due to biochemical degradation for instance, according to Beck (18). Sometimes techniques that avoid dietary problems may be used. For instance, the essentiality of amino acids in the black blowfly, *Phormia regina* Meigen, was determined by the radioactivity of each acid isolated from larvae fed glucose-U-¹⁴C or injected with L-glutamic acid-U-¹⁴C (20, 21). Vitamin requirements of certain insects were determined with chemicals analogous to specific vitamins (22, 23). King &

¹ The survey of the literature pertaining to this review was concluded in September 1961.

Sang (24) used an inhibitory analogue, aminopterin, to show that folic acid was involved in defective nucleic acid metabolism causing faulty oögenesis in *Drosophila melanogaster* (Meigen). However, for many nutritional investigations, analogues may not exempt endogenous related substances and so dietary needs are not always distinguished. Feeding techniques that enable omission or addition of dietary constituents are usually used to determine nutritional requirements of insects. The problems of concocting acceptable food media were discussed succinctly elsewhere (25, 26, 27).

According to Dougherty (19), insects are the only invertebrate Metazoa that have been raised axenically on chemically defined diets. The techniques used achieve such standardization of the insect as control over its environment and separation of its specific requirements from any possible host-symbiote relationship (19, 28). Sang (29), for example, used such techniques to determine the nutritional requirements of *D. melanogaster* and to probe metabolic capabilities. Nutritional defects can cause more or less characteristic symptoms of metabolic derangements (8).

More or less chemically defined diets, some in conjunction with axenic techniques, were developed for more than a dozen insect species, mostly of Diptera but including Coleoptera, Lepidoptera, Orthoptera, and others (3). Recent work developed such diets for the locust *Schistocerca gregaria* (Forskål) (30) and the cricket *Acheta domesticus* (Linnaeus) (31). Ito (32), and others cited by him (33), made noteworthy progress toward an unnatural diet for the silkworm, *Bombyx mori* (Linnaeus). These species include several plant feeders and one entomophagous parasite. None of them has very complex feeding habits, though *B. mori* is notably fastidious.

Physical and chemical feeding requirements are a great obstacle to nutritional research on certain insects. For instance, Scheel *et al.* (34) stated that inability to provide satisfactory feeding stimuli was the main reason, in their work, for poor growth of certain plant-feeding bugs on experimental diets. Other workers reported that changes in the physical properties of diets (35), immoderately high dietary osmotic pressures (36, 37, 38), and viscosity and particle size of food (39) forestalled optimum nutrition of different insects on otherwise adequate diets. Murray (40) found that the yellow mealworm, *Tenebrio molitor* Linnaeus, discriminated between different parts of its food and between different sizes of food particles, and that malnutrition did not lead to loss of appetite. Some nutrients have phagostimulant properties (41). Many substances that usually have no significant nutritive value are characteristic of certain plants and are responsible for host-plant specificity in many phytophagous insects (42, 43). For example, *B. mori* would feed on nutritionally inert agar impregnated with three attractants: β, γ -hexenol or α, β -hexenol, a methanol-soluble fraction, and a water-soluble fraction isolated from mulberry leaves (44).

NUTRITION

Present understanding of insect nutrition is founded on research that ranges from work on natural foodstuffs to that, during the last decade or so,

on chemically defined diets. It is however, limited, almost entirely to the needs of part, and not more than one generation, of a few representatives of Coleoptera, Diptera, Lepidoptera, and Orthoptera, mainly. Symbiosis and nutrient reserves are covert sources of nutriment that make it difficult to determine the nutritional requirements of many insects. Symbiotes usually occur in species that feed exclusively on substances such as plant sap, vertebrate blood, and certain stored products that are deficient in specific nutrients, but they also occur in cockroaches and a few other omnivorous insects (11). Nutrient reserves are passed from the female parent to the egg or are accumulated in the young and utilized in later stages of development in various insects (45, 46, 47, 48). Though there was much work with techniques to minimize or prevent the intervention of symbiotes, little was done until recently (45, 49) with insects depleted of nutrient reserves of particular substances. Dadd (50) pointed out the need to distinguish between essential nutrients and phagostimulants. For example, when poor growth results from omission of dietary components it is usually assumed that the components are essential nutrients, whereas poor growth may result from a reduction in feeding activity when the components omitted are phagostimulatory. Moreover, Luckey & Stone (51), in work on the sodium requirements of *Acheta domesticus*, pointed out that minute quantities of harmful substances may, in some cases, stimulate one or more metabolic systems but that stimulation by such substances does not qualify them as essential nutrients.

In general, there is evidence that nutritional requirements of young insects may vary with sex (52), change with development (18), and depend somewhat on the nutritional state of the parent (29), and also that deficiencies have the greatest effect on the smallest individuals (53). Requirements in the adult female may change during the course of egg formation and growth (54). Many workers pointed out the importance of proper balance between various nutrients for growth (29, 45, 55) and the importance of appropriate proportions of indigestible bulk material (50).

In contrast to the rather uniform qualitative requirements for amino acids, carbohydrates, lipids, vitamins, and minerals for growth of larvae, adult nutritional requirements vary widely. Adults of some species do not feed, some require carbohydrates only, while others need protein and other nutrients (7). These differences are related to the extent to which growth continues into the adult stage, in particular growth and development of the reproductive organs; and as these growth demands may differ with sex, nutritional requirements may likewise differ.

QUALITATIVE REQUIREMENTS

Proteins and amino acids.—Gordon (45) suggested that egg albumen is probably a better quality protein than casein for insects because albumen is most efficiently utilized in fast-growing small animals. Generally, the nutritional value of proteins or equivalent amino acid mixtures for growth (30, 55, 56, 57) and for reproduction (58) were found to differ with the species of insect. The relative order of protein quality for larvae of *Tribolium*

confusum Jacquelin du Val corresponded closely with that for the rat (57).

Usually with feeding tests the essentiality of an amino acid is determined by what happens to the insect when one acid is omitted from the diet. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are the essential amino acids for growth and development of the onion maggot, *Hylemya antiqua* (Meigen) (59), the pink bollworm, *Pectinophora gossypiella* (Saunders) (56), a beetle *Trogoderma granarium* Everts (60), and many other insects (4). The amino acid requirements of the German cockroach, *Blattella germanica* (Linnaeus), were thoroughly investigated by Gordon (45). Previously, Hilchey (52) had found that proline and serine were required only by the males, but Gordon did not confirm this. Dimond *et al.* (61) showed that the mosquito *Aedes aegypti* (Linnaeus) required the usual ten amino acids and glycine for growth, but Singh & Brown (38) found that cystine was essential and also that proline, hydroxyproline, and serine were needed, but not glycine. Such differences are probably due to variations in the nutrient reserves of the insects. Blackith & Howden (62), for example, found that the maternal diet of three species of locust influenced the fat content of the young, and that the amino acid content of hatchlings in the *gregaria* phase was greater than that of the *solitaria* for most of the acids present. Further insight into the nutritional status of amino acids may be gained by a multiple deletion technique. For example, a single deletion technique showed that proline, but not methionine, was essential for *Phormia regina* (63); a multiple deletion technique showed that either methionine or cystine, but not both, and either aspartic acid or glutamic acid were required (64). Glutamic acid was utilized in preference to aspartic acid, and cystine to methionine. Research with radioactive substances showed that *P. regina* slowly synthesized several amino acids, including proline (20, 21).

For reproduction, adult female *A. aegypti* require glycine and the same amino acids as for growth, but in one investigation (61) a cystine deficiency decreased fecundity whereas in another (38) it had no effect. The D-isomer of histidine was as effective as the L-form, the D-isomer of some other amino acids was not utilized, and that of still others was partly utilized (61). Dimond *et al.* (65) pointed out that results with *A. aegypti* should be applied with caution to other insects that differ physiologically.

Usually, however, the essential amino acids must be supplemented by a number of others for optimal growth (56, 60, 66). Certain iodoaminoacids and iodoproteins were shown to promote growth and development of the rice moth, *Corcyra cephalonica* (Stainton), but were not considered essential (67).

Carbohydrates.—Lipke & Fraenkel (2) stated that a carbohydrate may be nutritionally inert, satisfactory as a carbon source but unacceptable in a gustatory sense, or toxic. According to Pillai & Saxena (68), utilization varies with the species and depends on ability of the species to digest poly- and oligosaccharides to diffusible, absorbable forms and on the degree of absorbability of the molecule that is formed. The nutritive value of many carbohydrates was rated for growth (45, 69, 70), longevity (71, 72, 73), and fecun-

dity (74) in different species. Recent work is on *Bombyx mori* (75) and locusts (76). For example, the carbohydrate requirement in locusts is met by a number of hexoses, oligosaccharides, and sugar alcohols, but not by pentoses and certain hexoses; palatability was a negligible factor. In spite of specific variations concerning the utilization of sugars and related substances, glucose and fructose are usually well utilized by all insects, sorbose and galactose are not well utilized, and pentoses are used poorly or not at all. Adult *Drosophila melanogaster* utilized certain sugars best when the sugar had been present in the larval diet, and the degree of utilization differed with sex (72). Larvae of the housefly, *Musca domestica* Linnaeus, did not need carbohydrate (77).

Lipids.—Many fatty acids, including in some cases oleic, palmitic, stearic, capric, caprylic, caproic, and myristic acids, had no effect on or were detrimental to different insects (27, 77, 78). Apparently many insects synthesize all the fatty acids they require, both saturated and unsaturated. However, a few species, particularly amongst Lepidoptera, require exogenous unsaturated fatty acid. Certain moths require linoleic acid for larval growth (79), and for adult emergence, where linolenic acid also had an influence (78). *Blattella germanica* needed linoleic acid but deficiency symptoms became manifest only in the progeny of deprived parents (45). Symptoms of linoleic acid deficiency were shown recently in locust during the late stages of growth and development, particularly in the final molt and in wing formation (27, 30). The growth rate of an entomophagous insect *Agria affinis* (Fallén) was increased by exogenous fatty acids. The effect on growth may be rated as due to various combinations of oleic, palmitic, and stearic acid > oleic acid > palmitic acid > stearic acid; linoleic, linolenic, and arachidonic acid had no effect (80). Young (81) concluded that beeswax, paraffin wax, palmitic, stearic, and unsaturated 18-carbon acids enhanced growth of the greater wax moth, *Galleria mellonella* (Linnaeus), but related alcohols suppressed growth. Beck (82) concluded that beeswax and paraffin wax improved the physical consistency of the diet of this insect and that the beeswax might have contributed to the nutritional suitability, though it was not determined whether the beeswax contributed required nutrients or contained chemosensory feeding stimulants.

Insects require a sterol for growth and development. Cholesterol and its short-chain esters, 7-dehydrocholesterol, ergosterol, sitosterol, and certain other phytosterols are satisfactory for many species (83). Usually, however, cholesterol and sometimes a few other sterols are utilized best. For example, ergosterol was half, and sitosterol fully, as effective as cholesterol in the larvae of *Hylotrupes bajulus* (Linnaeus) (84). Recently the essentiality of a sterol and the selective utilization of a number of steroids were determined in *Bombyx mori* (32) and in two species of locusts (85). Cholesterol-deficient diets for adult *Musca domestica* decreased the hatch but not the total production of eggs (86). Levinson (87, 88) recently summarized work on sterol requirements and utilization, effects of sterol deficiencies in different insects, and other aspects of the subject. He (87) stated that the dependence of sterol

utilization on the feeding habits of insects can be interpreted in terms of the inability or ability of the various species to convert different sterols to cholesterol.

Nucleic acids.—Ribonucleic acid (RNA) usually increased the growth rate of different dipterous insects (2, 3, 7). Various components of RNA, alone or in combination, were effective to different degrees as substitutes for RNA (77, 89, 90). The requirement in *Drosophila melanogaster* was primarily for adenylic acid, essentially for adenine (90). *Agria affinis*, unlike *D. melanogaster* (91), utilized deoxyribonucleic acid (DNA) as well as RNA (92). It was said that the beetle *Sciobius granosus* Fähræus needed RNA for growth (93). Except for this claim, it appears that only the flies and mosquitoes need nucleic acids.

Vitamins.—The only vitamin discovered and isolated in entomology is carnitine, or vitamin B_T, and its importance has been shown only in several species of beetle of the family Tenebrionidae, according to Fraenkel (94). Singh & Brown (38) have claimed that *Aedes aegypti* requires carnitine. It is generally recognized that insects do not need fat-soluble vitamins. Most insects require biotin, choline chloride, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine for growth and development, though variations are found between species (1, 2, 3, 4, 6, 7). Recent work determined the vitamin requirements of *Musca domestica* (77, 95), *Calliphora vicina* Robineau-Desvoidy [= *C. erythrocephala* (Meigen)] (96), and locusts (97). Several vitamins were shown to increase fecundity and fertility in adult tephritids (98), but vitamins had no effect on adult *A. aegypti* (38). Though B₁₂ is active in purine synthesis (99), some insects did not require it in food (96, 100, 101). Growth of *Blattella germanica* ceased during the second generation on B₁₂-deficient diets (45). In *Corcyra cephalonica* it overcame the effects of a growth-inhibitory substance contained in raw soybean, but it was not a required nutrient (102). Until recently, ascorbic acid and inositol were of doubtful dietary importance in insects. However, Dadd (103) showed that ascorbic acid was essential to *Schistocerca gregaria*, and pointed out that it may be essential in some insects that, like some mammals, are unable to synthesize their requirements. Inositol was shown to be required by the boll weevil, *Anthonomus grandis* Boheman (104), two cockroaches (45, 105), a locust (97), and a cricket (31). *S. gregaria* needed inositol and β -carotene for normal pigmentation (27, 49, 97). This manifestation of a need for carotene is particularly noteworthy, because a need for fat-soluble substances other than sterols or fatty acids had not previously been satisfactorily established in insects.

Minerals.—*Drosophila melanogaster* required potassium, phosphorus, magnesium, and sodium, but not calcium except possibly in trace amounts (101). *Tribolium confusum* required magnesium and potassium, but not sodium or calcium (106). *Blattella germanica* probably needed manganese, copper, and zinc; requirements for other elements, such as sodium and calcium, could not be determined, but some were toxic under certain conditions (45). Zinc was required in *Tenebrio molitor* and manifestation of a

carnitine deficiency depended on the dietary content of zinc and potassium (107). Zinc can be toxic to *Corcyra cephalonica*, inhibiting growth and decreasing catalase activity; small amounts of copper restored catalase activity but did not improve growth (108, 109). Manganese seemed to have a beneficial effect upon fecundity in *Aplexis basizona* (Gravenhorst) (110). Brooks (111) found that magnesium was essential in *B. germanica* for bacteroid transmission from generation to generation; zinc acted as a synergist to magnesium. She stated, "the use of mineral salt mixtures designed for feeding vertebrates is illogical" in insect research. There is good evidence that she is right. The outcome of many investigations on mineral requirements of insects leaves much to be desired as it is difficult to control levels of elements, particularly trace amounts in food media. Fraenkel (107) has pointed out where caution must be taken in research on mineral requirements of insects.

Water and miscellaneous substances.—Many insects, both larval and adult, are known to drink water under certain conditions (112). Moreover, there is considerable evidence that various insects need unidentified substances. In some cases these may be peptides (64, 113). Lack of glutathione impaired the growth of *Aedes aegypti* (38). However, in some investigations where the question of unidentified substances was raised, it is conjectural whether malnutrition was due to lack of an unrecognized nutrient, to imbalances of recognized ones, or to unsatisfied feeding requirements.

QUANTITATIVE REQUIREMENTS

Friend *et al.* (114) stated, "The quantitative nutritional requirements and the effects of changes in the relative concentrations of nutrients are at present the most important phases in the study of insect nutrition and should be emphasized in the next few years." This view was supported by others (29, 55).

The quantitative work done is noteworthy, though much of it may not be very significant. Absolute quantitative requirements of the insect are not well determined because it is difficult to measure food intake. Quantitative data are usually expressed as the amount of a nutrient needed per weight or volume of food media. Thus, as Sang (29, 101) stated, estimates of minimal requirements give no direct measure of the quantities of each nutrient needed by a larva, but define only the relationship between particular requirements: a strain that has the same absolute requirements but that feeds faster than others would regularly show lowest minimal requirements, and vice versa. A satisfactory method of stating quantitative requirements is needed (29, 55). If quantitative requirements of the insect were expressed as the amount of nutrient eaten per day or, preferably, per caloric intake or unit of body weight, the data would have more meaning and usefulness to biologists (55). Sang (101) estimated the amount of each vitamin needed to produce one gram of *Drosophila melanogaster* pupae and proposed this criterion as a suitable way to compare requirements of insects that have different rates of food intake and that spent different times feeding (29).

The quantitative requirements, expressed as amount of nutrient per

unit of diet, were worked out for many insects (3, 7), including most of the essential nutrients for *D. melanogaster* (29, 101), many for *Blattella germanica* (45), and some for *Agria affinis* (55) and *Calliphora vicina* (115). In general, there appear to be optimal quantities of protein or amino acids (55, 101, 114), carbohydrates (55, 101), vitamins (101, 115), lipids (55, 80, 101), minerals (101, 106), and water (116) for each insect. The following are some examples: Increases in dietary protein increased fecundity in the oriental fruit fly, *Dacus dorsalis* Hendel (98). Normal development and metabolism of *D. melanogaster* depended on a satisfactory level of dietary protein: too little prolonged growth, whereas too much caused metabolic upset (101); this was more or less so for fructose, choline, and certain other nutrients (101). Growth rates of locusts, *A. affinis*, and of *Musca domestica* depended on sugar levels (76, 117, 118). Minimum dietary levels of different vitamins needed for good growth were determined for *D. melanogaster* (29, 101), *C. vicina* (115), and locusts (97). Amounts in excess of minimum optimal levels of vitamins generally had little or no effect on these insects, but excess of choline was detrimental to *D. melanogaster* (101). Inositol is a requirement of the American cockroach, *Periplaneta americana* (Linnaeus), because rates of survival, of growth, and of development increased with levels of inositol up to 40 to 80 mg/kg of diet (119). Growth rates of *A. affinis* (80), locusts (85), and *D. melanogaster* (101) were proportional to the supply of cholesterol up to a limit, but moderately excessive amounts had little or no additional effect. Certain fatty acids had similar effects on growth of *A. affinis* (80). Levels of RNA greater than half that needed for optimal growth of *A. affinis* decreased adult emergence (92). Dietary levels of a salt mixture and of various elements needed for optimal nutrition were determined in several insects, for example in *Tribolium confusum* (106).

The important quantitative requirements are the optimal proportional relationships, or balance, that are needed between dietary components, particularly essential nutrients (29, 45, 50, 55, 114). Gordon (45) has discussed the question of nutrient balance in insect nutrition most fully. It is recognized that a number of different but equally satisfactory ratios, or balances, between nutrients is possible (29, 45). According to Sang (29), strains could be selected from two insect species that would have the same quantitative vitamin requirements. He stated that balances between nutrients deserves the most investigation because here one approaches closest to an examination of metabolic processes. The significance of interrelations between nutrients determined in various insects include the following examples: Several workers showed that small variations in amino acid ratios had gross effects on growth and development of several insects (45, 55, 114). The locusts *Locusta migratoria* (Linnaeus) and *Schistocerca gregaria* differed markedly in their ability to grow on diets of amino acids or different proteins (30). Compositions of amino acid mixtures suitable for one species were unsuitable for another in many cases (30, 55, 56). The most suitable proportions of amino acids seem to resemble those found in the natural food of the insect (56). The amounts of essential amino acids needed by the honeybee,

Apis mellifera Linnaeus [= *A. mellifica* (Linnaeus)], expressed as ratios with the amount of tryptophan required, were very similar to those of other animals measured on the same basis (120). For egg-laying female *M. domestica*, the best dietary ratio of sucrose to protein determined was 7:1, that for males and non-laying females was 16:1 (121). Quantities of protein required for growth of *D. melanogaster* (29) and *M. domestica* (77) depended on carbohydrate levels. No interactions were found in *A. affinis* between glucose and an amino acid mixture equivalent to casein or between glucose and fatty acids (55). The presence of some substances may reduce the need for others, for example methionine and aspartic acid reduced the cystine requirement in *Phormia regina* (122). Lecithin protected *D. melanogaster* from the effects of excess choline (101). Folic acid, which could be partly replaced by purine, serine, and other substances, determined the need for RNA in *D. melanogaster* (29) and *M. domestica* (77). There was evidence of interactions between fatty acids in *A. affinis* (80).

RELATION OF NUTRITIONAL NEEDS TO METABOLISM

Nutritional research, particularly by means of feeding techniques sometimes furthered with chemical investigation, has illuminated some relationships between nutritional needs and metabolism. It is generally supposed that most nutrients function in insects much as they do in vertebrates. In *Drosophila melanogaster*, minimal vitamin requirements depend on metabolic rate and not on size (101). Of the vitamins required, only thiamine and riboflavin, which were involved in energy metabolism, were unaffected by protein levels (29). Metabolic rate, heat production, and food consumption increased in *Apis mellifera* at low temperatures (123). Carbohydrates promoted egg production in blood-fed females of the mosquito *Anopheles maculipennis* Meigen at suboptimal temperatures, but had no apparent effect at optimal temperatures (124). Hinton (125) listed the substances that are essential for *D. melanogaster*, those that are not absolute requirements and are probably synthesized, and those that may be substituted for others in nutrition. Because nutritional requirements depend on metabolic processes, determination of nutritional needs can lead to biochemical explanations of the capabilities for synthesis and of other processes, and, as Sang (29) remarked, nutritional techniques are not as blunt an instrument for doing this as is sometimes supposed. Johansson (126) stated, on evidence derived from the large milkweed bug, *Oncopeltus fasciatus* (Dallas), that nutrition and reproduction may be linked via the neuro-endocrine system. Valadares da Costa (127) found that defective nutrition in *D. melanogaster* reared on unsatisfactorily balanced diets caused embryonic variations, tumors, death of tissues, and phenocopies of mutant adults.

The specific role of the nutrient in question may be revealed when the manifestations of metabolic derangement caused by nutritional defects are localized in or are limited to a particular part of function of the insect. For example, Hinton *et al.* (128) found that immoderately high dietary levels of tryptophan caused deformed heads and tarsi, barlike eyes, and darkened

body and eye color in *D. melanogaster*. Kanehisa (129) pointed out the particularly close relationship in the same species between tumor formation and the tryptophan metabolic system involving eye color. Visible white deposits, presumably urates or uric acid, occurred in the legs, head, and other parts of *Periplaneta americana* reared on very high levels of protein (130). Larvae of *Corcyra cephalonica* on thiamine-deficient diets accumulated large amounts of pyruvic acid in their tissues (131), as do vertebrates. Pyridoxide deficiencies upset tryptophan metabolism in this insect. Abnormally colored feces are the first indication of this defect, and chemical techniques showed that this is due to a block in the conversion between kynurenine and 3-hydroxykynurenine, or 3-hydroxyanthranilic acid (132, 133). Tissues of biotin-deficient larvae of *C. cephalonica* did not desaturate palmitic acid and stearic acid as effectively as those of larvae fed biotin, and there was less fat and a greater accumulation of nitrogen in the tissues than in those of the biotin-fed controls (134).

Research on amino acid requirements showed that *Blattella germanica* needed no dietary cystine, methionine (135), or organic sulphur (45) in the presence of inorganic sulphates. It was shown, however, that intracellular symbiotes in this insect are responsible for sulphate utilization (136). Thus, *B. germanica* can utilize sulphates as a source of sulphur for cystine and methionine, and it can convert cystine to methionine (52, 135). Henry & Block (137) showed by chemical techniques how synthesis of cystine occurred in *B. germanica*, and what reactions cystine and methionine undergo to be convertible. The ability of insects to use sulphate in the synthesis of methionine was found to differ in various species (138). *Musca domestica*, in which sulphur metabolism resembles that of vertebrates, does not transform cystine to methionine (139). *Phormia regina* needs either cystine or methionine, but not both (122). Auclair (140) fed amino acids to *B. germanica*, and by semi-quantitative analysis found that the quality and concentration of amino acids in the blood depended on certain dietary amino acids and that results indicated the existence of deamination and transamination systems similar to those existing in mammals. Feeding tests with intermediary metabolites established the existence of a phenylalanine cycle in *B. germanica*, which explained why neither phenylalanine nor tyrosine is essential when sufficient tryptophan is fed, and why phenylalanine and tyrosine are nutritionally equivalent to each other in the cockroach (45). Possibly no such cycle occurs in *Aedes aegypti*, according to Gordon (45), because tyrosine cannot replace phenylalanine, and both phenylalanine and tryptophan are essential for growth of this mosquito (38). Earlier work (141) showed that neither tyrosine nor phenylalanine alone was essential, though omission of both in the presence of tryptophan prevented growth of the mosquito. Phenylalanine, an essential amino acid fed to the rice stem borer, *Chilo suppressalis* (Walker), was converted to tyrosine, a nonessential amino acid in this insect, and not to alanine (142). On the basis of feeding tests, Hinton (143) concluded that *D. melanogaster* had no ornithine cycle, as arginine was essential, citrulline only partly replaced it, and ornithine had no effect.

Similarly, ornithine was not an efficient precursor of arginine in *B. germanica* (45). Nutritional techniques showed relationships between glycine, serine, and folic acid and between protein supply, glutamic acid, and nicotinic acid in *D. melanogaster* (29). Sedee (115) fed N^{15} -labelled compounds to *Calliphora vicina* and found that this insect, like the rat, can utilize dietary ammonia for amino acid synthesis; that glutamic acid, alanine, and aspartic acid played a central part in protein metabolism of larvae; and that only leucine and valine entered into reversible transfer reactions, but that at least five amino acids did not exchange amino groups.

Treherne (144) concluded that sugars absorbed through the mid-gut of *Schistocerca gregaria* were converted to trehalose, which accumulated in the blood. To Candy & Kilby (145) trehalose appeared to be an important energy reserve in some insects: in *S. gregaria* it was synthesized in the fat body. Generally the sugars that caused an increase in glycogen in *Bombyx mori* caused an increase in trehalose (146). Hori (147) surmised that some physiological mechanism maintained a relatively constant level of blood trehalose in *B. mori* during starvation. Wyatt & Kalf (148) found that trehalose made up 90 per cent of the blood carbohydrate in some species. In *Agria affinis* larvae, however, glucose comprised at least 80 per cent of the total (149). Blood carbohydrates in this insect were not changed by diets containing from 0 to 1 per cent glucose, though they were increased, but not changed qualitatively, by increasing the glucose content of the diet to 2 per cent (149).

The absolute dietary requirement and broad specificity of sterols for insects, the limited substrate specificity of *P. americana* "cholesterol esterase", and the incorporation *in vivo* of the C^{14} from acetate into the sterol molecule by the cockroach indicated to Casida *et al.* (150) that insects may be able to cleave the sterol side chain and resynthesize the isooctyl side chain of cholesterol. Several analogues of cholesterol containing short side chains were synthesized and fed to the Levant housefly, *Musca vicina* (Macquart), but these unnatural sterols were not as effective as cholesterol for promoting growth (151). To some extent, larvae of *M. vicina* converted dietary sitosterol into a sterol that is nutritionally equivalent to cholesterol or 7-dehydrocholesterol in *Dermestes* larvae (152). Clark & Bloch (153) found that *B. germanica* grew with ergosterol as the sole source of sterols and, by subsequent biochemical techniques, they found that the 5,7-diene ergosterol was demethylated and reduced at the Δ^7 position to form 22-dehydrocholesterol. They (154) showed that ergosterol was inactive in the hide beetle, *Dermestes maculatus* De Geer [= *D. vulpinus* Fabricius]. Evidently *D. maculatus* lacked the necessary mechanism for modifying ergosterol (153). Feeding sterol precursors, such as mevalonic acid, squalene, and lanosterol, neither fulfilled nor reduced cholesterol needs of *D. maculatus* (155). This suggested to these authors (155) that the pathways of cholesterol biogenesis are blocked at various stages in this insect and not interrupted at the squalene stage only, as previously reported (156). Robbins *et al.* (157) pointed out that a metabolic pathway for cholesterol in the cockroach is its

conversion to a 5,7-diene, which behaves like 7-dehydrocholesterol, and that this conversion has also been found to occur in *M. domestica* (158) and in *Tribolium confusum* (159). Moreover, they found that cholesterol was efficiently utilized in *B. germanica* and that the sterol antagonist cholesteryl chloride caused only about 10 per cent decrease in cholesterol utilization. Levinson (87, 88) pointed out that larvae may be divided according to sterol utilization: into phytophaga, which are capable of converting C_{28-29} sterols of their food plants to cholesterol, and carnivora, which are adopted to cholesterol utilization only. *B. germanica* and *C. vicina* shorten the side chains of dietary ergosterol and β -sitosterol by, respectively, demethylation or deethylation (88). Though other events have been described in a broad variety of species, Levinson (87) stated that work is urgently needed that would lead to understanding of the mechanisms. He pointed out, moreover, that cholesterol is required for larval growth and for oögenesis and that its function resembles that of the juvenile hormone, neotenin, in insects. Cholesterol may act, therefore, as a precursor of steroid hormones, in addition to its structural and anti-infective roles (87). The significance of the 5,7-diene, or provitamin D, structure in insects—its formation, function, and metabolism—merits further investigation (157).

There is evidence that insects differ in the way they utilize nucleic acids. *D. melanogaster* (90) and *A. affinis* (92) develop without dietary nucleic acids but grow faster with RNA; however, *A. affinis* can also use DNA. Sang (90) used feeding techniques to show that the requirement for RNA in *D. melanogaster* was for adenylic acid but that other substances may promote growth; some, together with adenylic acid, were equivalent to RNA; the pyrimidines and their nucleosides and nucleotides were not utilized alone (101). Hinton's (89) results differed in but a few respects from Sang's (90, 101). RNA determined the need for folic acid, which acts in the conversion of uridine to thymidine (29). Feeding DNA overcame the action of aminopterine in *D. melanogaster* (160). Both RNA and DNA in the diet of *C. cephalonica* overcame growth inhibition, and B_{12} overcame mortality caused by zinc toxicity (109). The explanation for the behavior of B_{12} was that it may be involved in nucleic acid synthesis in the larvae, particularly in view of the influence of nucleic acids on zinc toxicity. McMaster-Kay & Taylor (161) fed adenine 8- C^{14} to *D. melanogaster* and found that RNA- C^{14} and DNA- C^{14} appeared in chromosomal nucleic acids in the salivary glands, and that RNA- C^{14} was utilized in the synthesis of DNA.

Dadd (162) stated that locusts apparently differ from most insects in being unable to synthesize adequate amounts of ascorbic acid. In the absence of ascorbic acid from the diet, a reducing substance, presumably ascorbic acid, normally present in the blood and functioning in relation to the molting cycle, failed to appear. He concluded that, in locusts, ascorbic acid played some part in the processes occurring at the molt, particularly in the hardening and melanization of cuticle. Work done with homogenates of *P. americana* showed that various sugars were utilized as precursors in the biosynthesis of

ascorbic acid: mannose produced the most and xylose the least (163). Briggs (164) concluded that the tyrosine oxidase system in *Blattella conjuncta* (Walker) was activated by ascorbic acid, also by folic acid, but most readily by additions of ascorbic acid, glutathione, and pyridoxal phosphate together. Supplementation of the diet of *C. cephalonica* with L-cystine, DL-tryptophan, and several B vitamins, affected growth and fat synthesis in specific ways that were indicative of the role played by each vitamin in fat metabolism (165). Dadd (49), who was the first to determine the effects of carotene on an insect, discussed the relation of these effects on blood pigments and body color to the various hypothesis of humoral regulation of the solitary and gregarious phases that occur in the locust.

GENETIC BASES OF NUTRITIONAL NEEDS

Different workers have found that nutritional requirements differ within certain species of insects (58, 166, 167, 168). According to Hinton & Dunlap (169), there is a genetic basis for nutritional requirements, as metabolites may become requirements depending on genetic constitution. The most fundamental work is on *Drosophila melanogaster*. Strains were demonstrated in which many variations occur in needs for RNA, its components, and several amino acids (125, 169). In some the sex chromosome was involved in adenine synthesis, so that RNA was required. Another strain gradually changed its adenine requirement: originally with RNA in the larval diet no adults emerged; after many generations about half do so (125). No remarkable differences in nutritional requirements were found between several "standard" strains (29).

Sang (99) determined differences in the nutritional requirements of *D. melanogaster* in relation to heterosis, or hybrid vigor. He found, for example, that the minimal requirement for nicotinic acid in a hybrid was intermediate between the requirements of its parental strains, though in some other cases the hybrid needed more or less of a given vitamin than the parental strains. He concluded that hybrids, which either use more vitamins or make better use of them, have more effective enzyme systems, and hence are biochemically more efficient than their parental strains.

According to Sang (29), differences between the needs of strains of *D. melanogaster* provide a selective advantage and indicate the likelihood of adaptation to new nutritional environments within and between species. Nutritional differences that result from evolution may be most apparent at the level of metabolism; hence, differences in the utilization of substances and the peculiarities of adaptation to particular environments are significant (29).

ECOLOGICAL SIGNIFICANCE OF NUTRITIONAL FACTORS

Intricate relations exist between insects and their food plants and, perhaps no less so, their animal hosts. Often, however, the effects of different foodstuffs cannot be attributed primarily to nutrition, as feeding behavior

and other nonnutritional factors are involved. With many phytophagous insects, host selection is directed by tactile, olfactory, gustatory, and other stimuli (41, 42, 43). Feeding activities and larviposition rates of *Aphis fabae* Scopoli were found to decrease on water-deficient leaves (170). The European corn borer, *Ostrinia* [= *Pyrausta*] *nubilalis* (Hübner), feeds mostly on corn tissues of highest sugar content, though occurrence of high larval mortality was not caused by inadequacies of nutrients but by toxic substances in the plant tissues (18, 171, 172).

It has been proposed either that nutritionally unimportant stimuli are predominantly responsible for food preferences or that host selection is a dual discrimination reaction that involves nutrition. Fraenkel (43), for example, supported the first alternative, for which there is good evidence that food specificity of insects is solely based on the presence or absence of compounds in plants that serve as repellents to insects in general and as attractants to those few which feed on each plant species. Though there is much evidence for this point of view, several works showed that nutrients probably play an important role in the selection of food plants (41).

Undoubtedly nutrition does play a significant role in the natural control of insects, though its importance is difficult to evaluate. Only a brief discussion of how nutrition is implicated in ecological problems can be given here. Many investigators have shown that the susceptibility or resistance of insects to insecticides is affected by the kind and quality of food eaten (by insects) and by the nutriment stored as reserves in their body tissues (173). Bergold (174) concluded that the kind of food eaten by the insect seems to play an important part in the susceptibility of insect populations to virus diseases. The nutrition of host plants, which is affected by the application of fertilizers, nutritional sprays, and pesticides, can in turn affect the nutrition of phytophagous insects (17).

Slight differences in food have various effects on insects. Smith (175) found that the sex ratio, reproductive rate, size, and survival rate of the female as compared with the male varied in several species of hymenopterous parasites with the food plant of their host, the California red scale, *Aonidiella aurantii* (Maskell). Atwal (176) found that a number of metabolic and anatomic abnormalities occurred in the diamondback moth, *Plutella maculipennis* (Curtis), when the proportions of nutrients in the leaves of the host plant were upset, and concluded that normal activities of this insect depend on the balance of nutrients in its food plant. When *Agria affinis*, reared on chemically defined diets, was parasitized by *Aphaereta pallipes* (Say), the emergence of the parasites could be related to the ratio of glucose to amino acids in the diets of the host; effects due to differences in age of the host and to differences in osmotic pressure of host blood on the different diets could be ruled out (177).

According to Beck (18), however, little evidence was obtained prior to 1956 to indicate that varietal resistance in plants was attributable to differences in content of nutritionally important substances, though nutritive

factors contributing to host-plant adequacy were identified. Auclair (178) suggested that adverse effects on insect development might be caused in some cases by unsatisfactory nutritional value of the food plant in question. This seemed to be the explanation for varietal resistance in peas, as varieties most susceptible to the pea aphid, *Acyrtosiphon pisum* (Harris), contained more nitrogen and less sugar than resistant varieties (179). Resistant varieties usually contained the lowest concentrations of free and total amino acids (180). It was concluded that such concentrations reduced the rate of growth and reproduction of the aphid and so contributed to the resistance of these varieties. In similar investigations, Marble *et al.* (181) found that one strain of alfalfa susceptible to the pea aphid and moderately resistant to the spotted alfalfa aphid, *Therioaphis maculata* (Buckton), contained more total free amino acids than a strain resistant to the pea aphid in field, but not in greenhouse, trials. Thus it seemed to these workers that the choices of host plants were opposite in relation to amino acid contents and that the pea aphid and alfalfa aphid had different nutritional needs. It was also found that the amino acid content of alfalfa was affected by environment. Though no conclusive differences in amino acid composition were found between resistant and susceptible alfalfa plants, only the aphids feeding on susceptible plants contained β -alanine and ethanolamine.

An important aspect of the problem is the efficiency with which food is converted to energy and to body tissues. Efficiency depends largely on the balance of nutrients and helps to determine the nutritional value of a food. It was shown that the efficiency of conversion of wheat plant into body tissues of the migratory grasshopper, *Melanoplus bilituratis* (Walker), was intermediate between that for the oat plant and that for western wheat grass (182); and that the consumption, excretion, and increase in dry matter and nitrogen in body tissues of the pale western cutworm, *Agrotis orthogonia* Morrison, fed on two varieties of wheat, differed somewhat (183).

Much of the work on insect nutrition is aimed at determining the optimal dietary requirements (1, 2, 3, 4, 6, 7). Gordon (45) stated, however, that the ecologically significant question is not "what is an optimal diet for this animal?" but "what is the most deficient and unbalanced diet that the animal can tolerate without drastic reduction of its rate of growth and reproduction?" He pointed out that "high food efficiency" increases the number of organisms that can be produced on a given amount of food. Hence, selection pressure occurs that favors genotypes that can utilize a given food source with maximum efficiency.

Much more critical investigations are necessary to explain the role nutrition plays in the control of insects. In almost all work to date on the problem of varietal resistance in plants, very little, if anything, is known about the nutritional requirements of the insects involved, or about the precise chemical composition of the plant, or about both in some cases. The best exception to this is the work by Beck (18) and his associates on the resistance of the corn plant to *O. nubilalis*. Auclair (178) pointed out the need

for information on the quantitative nutritional needs of phytophagous insects, and for work in which chemically defined diets are used to investigate the problem of resistance in plants. Painter (184) states that not enough emphasis has been placed on the use of resistant varieties, particularly resistant and susceptible isogenic pairs. What seems most necessary is work in which precisely detailed qualitative and quantitative analysis of the composition of the host plant in terms of nutrients is related to the precise qualitative and quantitative requirements of the insect concerned.

CONCLUDING REMARKS

Little mention has been made in this review of work on natural foodstuffs that in many respects has heuristic importance, though little chemical detail. Nutrition is best understood in chemical terms as it is basically a chemical subject. Investigations of insect nutrition on this basis are performed with synthetic diets and by techniques that enable precise determinations to be made. Such techniques have helped to gain an insight into quantitative requirements within the last decade. Notable work recently on qualitative requirements includes proof that several insects require dietary sources of inositol and that at least one species needs exogenous ascorbic acid and β -carotene.

The volume and variety of work on insect nutrition is increasing. Because many insect problems have resisted solution, many workers have sought knowledge of nutrition as basic for investigations of the vital processes and the various functions that occur within the insect. Thus, there seems to be a trend toward nutritional investigations that can establish relations between insect nutritional requirements and biochemistry, physiology, and related subjects. Nutritional probing cast some new light on significant interrelationship between many nutrients and on metabolic pathways and capabilities of synthesis. Some work helped demonstrate the genetic bases of variations in nutritional requirements. Symptoms of deranged metabolism are often manifest in tissues and microstructures, and in biochemical and physiological processes that may signal the importance of a given nutrient in vital roles. These symptoms are often overlooked. Work is needed on a comprehensive basis in insect nutritional research so that light may be thrown on the pathways of nutritional evolution. Very little is known about the nutrition of insects in a number of taxonomic orders, of highly specialized parasitic species, or of species that have unusual feeding habits and life-histories. Moreover, the possibility that insect resistance of plants may be related to nutritional factors important to insects is an economic problem that awaits thorough investigation. This problem cannot be solved by nutritional techniques alone. One may speculate that control of insects may be obtained by development of plant varieties nutritionally inadequate for the pest insect concerned. However, the effectiveness of this stratagem as a potential method of pest control could be reduced by the insect as the nutritional requirements of an

insect can vary within a species which would probably adapt itself to the new varieties of the food plant.

Heller (185), writing on the biochemistry of insects, stated that generalizations are difficult. This also applies to nutrition, as it seems that virtually every species presents a paradox to our understanding of the subject.

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